FACTORS AFFECTING THE ASSOCIATION-DISSOCIATION EQUILIBRIA OF PROTEINS WITH SPECIAL REFERENCE TO STUDIES ON β-LACTOGLOBULIN AND HAEMOGLOBIN

A thesis submitted for the Degree of

Doctor of Philosophy

in the

Australian National University

by

PETER ADRIAN BAGHURST

January, 1973.

The research work described in this thesis was undertaken in the Russell Grimwade School of Biochemistry, University of Melbourne, between March, 1970 and January, 1971, and in the Department of Physical Biochemistry in the John Curtin School of Medical Research, Australian National University, between February, 1971 and January, 1973. All the experimental results reported in the text were obtained by the author alone, while theoretical considerations of ligand binding to a self-associating acceptor, in Chapter I, and of mass migration patterns, in Chapter III, were made in collaboration with Professor L. W. Nichol.

ACKNOWLEDGEMENTS

The author is deeply indebted to Professor L. W. Nichol, for his guidance and encouragement in the inception and subsequent development of this thesis, and to Dr. W. H. Sawyer for his joint supervision of that part of the work which was carried out at the University of Melbourne.

Sincere thanks are also due to Dr. D. J. Winzor and Mr. R. J. Richards for communicating to the author, the results of their chromatographic study of the acid-expansion of bovine serum albumin, and to Dr. K. O'Dea for a valuable discussion on the possible action of biological effectors. The Red Cross Blood Bank at the Canberra Community Hospital kindly consented to assist the author in collecting samples of his blood.

Financial support from the Commonwealth Postgraduate Research Award Committee and the Australian National University is gratefully acknowledged.

CONTENTS

INTRODUCTION

This work is primarily concerned with aspects of the phenomenon of protein polymerisation (Reithel, 1963; Nichol, Bethune, Kegeles and Hess, 1964; Cann, 1970; Nichol and Winzor, 1972). The current growth of interest in this subject is due, in part, to the experimental finding that a wide variety of proteins exist in solution as equilibrium mixtures of forms of different molecular weights. While such interactions have been the subject of intensive investigation only recently, their existence was demonstrated more than forty years ago, by sedimentation velocity studies on the respiratory proteins, haemoglobin and haemocyanin (Svedberg and Fåhræus, 1926; Svedberg and Heyroth, 1929; Svedberg and Hedenius, 1934). Subsequently, Longsworth and MacInnes (1942) recognised the importance of classifying association-dissociation reactions in terms of their rates of equilibration. The behaviour of slowly equilibrating systems in the mass migration processes of sedimentation velocity and moving boundary electrophoresis was explicable in terms of theories developed for the migration of non-interacting mixtures; while that of rapidly equilibrating systems was only imperfectly understood (Field and Ogston, 1955) until the theoretical treatment of Gilbert (1955, 1959) became available. The latter systems, in which equilibrium is rapidly established following the application of a constraint, (for example a centrifugal field or a temperature variation) are the concern of this work.

Recent developments in the study of association-dissociation reactions have proceeded along three major pathways. The first of these has been concerned with the

thermodynamic description of the equilibria involved. Experimental methods which have been used for this purpose may be broadly classified into the mass migration procedures of sedimentation velocity, moving boundary electrophoresis, and frontal gel chromatography, and the equilibrium methods of sedimentation equilibrium, light scattering and osmotic pressure. Application of these methods and their related theories has resulted in the evaluation of equilibrium constants (Gilbert, 1955, 1959), non-ideality coefficients (Adams, 1965), enthalpy changes (Jeffrey and Coates, 1966) and volume changes (Howlett, Jeffrey and Nichol, 1970, 1972). Research in the second area of enquiry has sought to determine the bond types and the specific amino acid residues involved in certain polymerisations. X-ray diffraction analysis (Perutz, 1970), chemical modifications of the protein (Creeth and Nichol, 1960; Armstrong and McKenzie, 1967), variation of environmental conditions (Aune, Goldsmith and Timasheff, 1971) and comparative studies on genetic variants (McKenzie, Sawyer and Smith, 1967) have all contributed to the understanding of the chemistry of particular interactions. In the third field of endeavour, the possible biological significance of association-dissociation reactions has been explored, with particular emphasis on sigmoidal effects observed in the binding of small molecular weight compounds (ligands) to protein acceptors (Monod, Wyman and Changeux, 1965; Nichol, Jackson and Winzor, 1967; Frieden, 1967; Langerman and Klotz, 1969). It is interesting to note that in 1912, Douglas, Haldane and Haldane suggested that sigmoidal binding curves of oxygen to human haemoglobin might conceivably arise

as a direct consequence of self-association of the protein.

A major purpose of this work is to make specific and more detailed comment on each of these three areas of development, and to point out relationships between them. Chapter I commences with a review of the models which have been proposed to account for sigmoidal binding curves. Attention is focussed on those models involving acceptors which are self-interacting, a term which encompasses not only polymerisations but also isomerisations. It is shown that preferential binding of a ligand to one isomeric or polymeric state of an acceptor (Monod *et al.*, 1965; Nichol *et al.*, 1967a) is a necessary but not sufficient condition to ensure a sigmoidal response, which is characterised by a binding curve exhibiting a point of inflection. The boundaries of regions within which a given system will exhibit sigmoidality are defined in terms of the relevant equilibrium constants and numbers of binding sites. The value of the equilibrium constant(s) governing the self-interaction of the acceptor is found to be particularly significant. Since this constant is, in general, a function of temperature, pressure and the activities of other species in the solution (including the solvent), it follows that environmental parameters may determine whether a given acceptor system lies within, or without, a domain of sigmoidality. Thus, there is a need, not only to measure constants of self-interaction, but also to determine their variation with, for example, temperature, pressure, pH and ionic strength.

Chapter II is concerned with the use of mass migration methods in the determination of protein association constants.

It reviews the pioneering theoretical work of Gilbert and coworkers, and introduces the most recently developed migration method of frontal gel chromatography (Winzor and Scheraga, 1963). Application of the theory is illustrated with studies on the A variant of bovine β-lactoglobulin under conditions in which the dimeric protein associates to form an octamer. Measurements are made over a range of temperatures in both aqueous and deuterium oxide buffers, and the results are used to draw inferences concerning the nature of the intermolecular bond types. Particular attention is given to the suggested use of deuterium oxide (Lee and Berns, 1968) in detecting hydrophobic interactions. While the biological significance of β-lactoglobulin is, as yet, unknown (Timasheff and Townend, 1969) the protein serves as a useful model for studying self-interacting systems, and in Chapter III it is used again to test a differential chromatographic technique for comparing the extents of association of a protein in two different environments. The establishment of such a rapid, direct method of comparison is important if a wide variety of protein association-dissociation reactions, and the factors which affect them, are to be studied.

Chapter IV is concerned with a biologically important acceptor system, normal adult haemoglobin, and the small molecular weight effectors 2,3-diphospho-D-glyceric acid and adenosine 5'-triphosphate. These organic phosphates affect the shape of the oxygen binding curve of haemoglobin to such a degree, that their existence in the erythrocyte is vital to the release of oxygen to the tissues (Benesch and Benesch, 1969). Their effects on the dimer $(\alpha\beta) \rightleftharpoons$ tetramer $(\alpha\beta)_2$

association equilibrium of haemoglobin at pH 5.4 and on the separated α and β polypeptide chains at the same pH are studied by conventional mass migration procedures, the new differential chromatographic technique, and sedimentation equilibrium. Connection is made, therefore, with the material of Chapters II and III and with the section in Chapter I dealing with the action of effectors in perturbing acceptor self-interactions. While the acid dissociation of haemoglobin is unlikely to be of physiological significance (Perutz, 1970a) it nevertheless serves as a useful probe for investigating the action of biologically important organic phosphate effectors.

In summary, the aims of this thesis are threefold:

(1) by extending theory describing the binding of a ligand to a self-interacting acceptor, to stress the necessity of characterizing protein associations in a variety of environments;

(2) to develop a method of differential chromatography which may prove to be generally useful for making a direct comparison between the extents of association, or between the conformational states of a protein in two different environments, and

(3) to comment specifically on the effects of variations in selected environmental parameters on the association-dissociation equilibria of β-lactoglobulin and haemoglobin.

It is hoped that the specific information obtained with these proteins will assist in their complete characterization, and that the general theoretical results may find use in the

study of the increasing number of proteins which are known to self-interact.

CHAPTER I

THE BINDING OF LIGANDS TO ACCEPTOR SYSTEMS: FACTORS WHICH DETERMINE THE SIGMOIDAL RESPONSE

SECTION A: A REVIEW OF POSTULATED BINDING EQUATIONS

In a study of the reversible binding of a ligand to an acceptor, the equilibrium concentration of unbound ligand c_S (g/l) may be determined experimentally, using, for example, the methods of equilibrium dialysis or frontal gel chromatography (Klotz, Walker and Pivan, 1946; Myer and Schellman, 1962; Nichol, Jackson and Smith, 1971; Cooper and Wood, 1968). If $\bar{c}_S$ and $\bar{c}_A$ are the known total weight concentrations of ligand and acceptor, respectively, in the mixture, a binding function, r, may be evaluated, where r is defined as the number of grams of ligand bound per gram of acceptor (Klotz, 1953; Weber, 1965)

$$r = \frac{\bar{c}_S - c_S}{\bar{c}_A} \qquad \text{(I-1)}$$

A binding curve is obtained by plotting values of r against the corresponding values of c_S found in a series of experiments in which $\bar{c}_S$ is varied while $\bar{c}_A$ is held constant.

Frequently, the experimentally obtained binding curve is interpreted in terms of a theoretical relationship between r and c_S, called a binding equation, which is derived on the basis of an assumed model. Basically two types of model have been proposed to explain sigmoidal binding curves. The first involves the binding of a ligand to an acceptor which exists as an equilibrium mixture of different isomeric (Monod, Wyman and Changeux, 1965) or polymeric (Nichol, Jackson and Winzor, 1967; Frieden, 1967; Klapper and Klotz, 1968) forms. The second encompasses situations in which the binding of a ligand to a single form of the acceptor induces a conformational change in the

acceptor and alters the apparent affinity for the ligand at distant binding sites. (e.g. Koshland, Némethy and Filmer, 1966; Koshland and Neet, 1968). Each one of these models and the binding equations derived from it will now be considered.

1. The binding of ligand to a self-interacting acceptor.

(a) Derivation of the binding equation

Consider first the simplest self-interaction of an acceptor in which two forms coexist in equilibrium, $nA \rightleftharpoons C$. The equilibrium constant X is defined as

$$X = [C]/[A]^n \qquad (I-2)$$

where [ ] denote molar concentrations. When n = 1, the molecular weights of A and C are identical ($M_A = M_C$) and the isomerisation constant, X, is dimensionless. When n > 1, the reaction is called a polymerisation ($nM_A = M_C$) and the association constant is expressed in moles^{1-n}.litres^{n-1}. The binding of a ligand, S, to an acceptor A with p available binding sites per molecule, may be represented generally by $AS_{i-1} + S \rightleftharpoons AS_i$ (i = 1,2,...,p) where AS_0 is taken to mean unbound A. Similarly, the multiple binding of S to C is described by $CS_{j-1} + S \rightleftharpoons CS_j$ (j = 1,2,...,q). The related binding constants are defined on a molar scalar as

$$L_i = [AS_i]/[AS_{i-1}][S] \qquad (I-3a)$$

$$M_j = [CS_j]/[CS_{j-1}][S] \qquad (I-3b)$$

Klotz (1946) originally noted that the formulation of binding equations is considerably simplified if the sites on

a particular acceptor species are regarded as equivalent and independent, so that all the L_i may be expressed in terms of a single intrinsic association constant K_A, and similarly, all the M_j may be expressed in terms of a K_C. The concept of equivalent and independent sites was used by both Monod *et al.* (1965) and Nichol *et al.* (1967a), and it constitutes a basic difference between the model proposed by these workers, and that postulated by Koshland and coworkers.

The terms 'equivalence' and 'independence' may be understood by considering a single acceptor, A, possessing two binding sites (p = 2). An equilibrium mixture of acceptor and ligand will contain unbound A and S and the complexes -A-S, S-A- and S-A-S. The two sites are regarded as *equivalent* if the same equilibrium constant describes the binding of a single ligand molecule to either site, such that

$$[S\text{-}A\text{-}] = [\text{-}A\text{-}S] = K_A[A][S] \qquad \text{(I-4a)}$$

The sites are said to be *independent* if K_A also governs the binding of the second molecule of ligand to the remaining site, irrespective of the pathway of formation of S-A-S

$$[S\text{-}A\text{-}S] = K_A[S\text{-}A\text{-}][S] = K_A[\text{-}A\text{-}S][S] = K_A^2[A][S]^2 \qquad \text{(I-4b)}$$

In general, when the p sites on A are equivalent and independent

$$[AS_i] = \alpha_i[A](K_A[S])^i \qquad \text{(I-4c)}$$

where α_i is the number of ways of binding i molecules of S to A,

$$\alpha_i = \binom{p}{i} = \frac{p!}{i!(p-i)!} \qquad \text{(I-4d)}$$

and $[AS_i]$ is the sum of the concentrations of all the possible forms of AS_i some of which, by symmetry, may be indistinguishable from one another. It follows from equations (I-3a), (I-4c) and (I-4d) that the stepwise association constant, L_i, is related to the intrinsic binding constant, K_A by the equation (Klotz, 1946)

$$L_i = \alpha_i K_A/\alpha_{i-1} = K_A(p-i+1)/i \qquad \text{(I-5)}$$

For the case in which C coexists with A in an equilibrium mixture, it follows by similar reasoning that

$$[CS_j] = \alpha_j [C](K_C[S])^j \qquad \text{(I-6a)}$$

$$\text{and} \quad M_j = K_C(q-j+1)/j \qquad \text{(I-6b)}$$

provided the q sites on C are equivalent and independent.

The total weight concentration of acceptor, $\bar{c}_A$, is given by

$$\bar{c}_A = M_A \sum_{i=0}^{p} [AS_i] + nM_A \sum_{j=0}^{q} [CS_j]$$

$$= M_A[A] \sum_{i=0}^{p} (K_A[S])^i \alpha_i$$

$$+ nM_A[C] \sum_{j=0}^{q} (K_C[S])^j \alpha_j \qquad \text{(I-7)}$$

The summations in equation (I-7) are recognised as binomial expansions and the equation may be rewritten as

$$\bar{c}_A = M_A[A](1+K_A[S])^p + nM_A[C](1+K_C[S])^q \qquad (I-8)$$

Similarly, the concentration of *bound* ligand is given by

$$\bar{c}_S - c_S = M_S \sum_{i=1}^{p} i[AS_i] + M_S \sum_{j=1}^{q} j[CS_j] \qquad (I-9)$$

where M_S is the molecular weight of the ligand. Combining equations (I-4c), (I-6a) and (I-9) and substituting $i\alpha_i$ with $p\beta_i$ where $\beta_i = \binom{p-1}{i-1}$ yields

$$\bar{c}_S - c_S = M_S[A] \sum_{i=1}^{p} i(K_A[S])^p\alpha_i + M_S[C] \sum_{j=1}^{q} j(K_C[S])^q\alpha_j$$

$$= M_S pK_A[S][A](1+K_A[S])^{p-1} + M_S qK_C[C][S](1+K_C[S])^{q-1} \qquad (I-10)$$

The binding equation for a self-interacting acceptor is obtained by combining equations (I-1), (I-2), (I-8) and (I-10)

$$r = \frac{M_S pK_A[S](1+K_A[S])^{p-1} + M_S qK_C[S]X[A]^{n-1}(1+K_C[S])^{q-1}}{M_A(1+K_A[S])^p + nM_A X[A]^{n-1}(1+K_C[S])^q} \qquad (I-11)$$

(b) A discussion of the binding equation

As Nichol *et al.* (1967a) have noted, equation (I-11) simplifies to a form describing a rectangular hyperbola,

$$r = \frac{M_S p K_A [S]}{M_A (1+K_A [S])} \qquad \text{(I-12)}$$

under two sets of conditions. The first stipulates that X = 0, i.e. ligand binds to equivalent and independent sites on a *single* form of the acceptor A; the situation originally treated by Klotz (1946). The second set of conditions requires that q = np and $K_A = K_C$, i.e. no sites are lost or gained, and the affinity for ligand is unperturbed by the process of self-interaction. In the event that q ≠ np and/or $K_A \neq K_C$, deviations from the form of a simple rectangular hyperbola must arise. Monod *et al.* (1965) considered a particular set of examples of preferential binding of ligand to one form of the acceptor. In detail, n was taken as unity (an isomerisation), q = p, $\beta = K_C/K_A$, $\alpha = K_A[S]$ and $r_m = M_A r/M_S$ (the molar binding function). In these terms, equation (I-11) becomes

$$r_m = \frac{p\alpha(1+\alpha)^{p-1} + p\alpha\beta X(1+\alpha\beta)^{p-1}}{(1+\alpha)^p + X(1+\alpha\beta)^p} \qquad \text{(I-13)}$$

which is identical to equation (2) of Monod *et al.* (1965). Provided β ≠ 1 (preferential binding), equation (I-13) describes a binding curve which deviates from the form of a rectangular hyperbola to an extent determined by the values of X, β and p. In numerical examples for which β = 0 (exclusive binding to the A form), Monod *et al.* (1965) have shown (their Figure 1(a)) that the binding curves are sigmoidal, and that the effect becomes more pronounced as X increases. The interesting conclusion to be drawn from these, and other examples, is that sigmoidality is favoured

by a large initial excess of the isomer with the lower affinity for the ligand.

When the acceptor polymerises ($n > 1$), equation (I-11) is identical to equation (7) of Nichol *et al.* (1967a) who also concluded, through numerical examples, that pronounced sigmoidality of the binding curve was favoured by a large initial excess of the less active polymer. Preferential binding of ligand to a polymerising system may arise as a result of either a lack of conservation of binding sites ($q \neq np$) or a change in the affinity ($K_A = K_C$). The preferential binding of N-acetylglucosamine to the monomeric form of lysozyme (Howlett and Nichol, 1972a) provides an example of the former situation, and the binding of the competitive inhibitor phenylpropiolic acid to the monomeric and dimeric forms of α-chymotrypsin (Nichol, Jackson and Winzor, 1972) is an example of the latter.

It might appear, at first sight, that the consideration of the dual condition, $q \neq np$ and/or $K_A \neq K_C$ is the only new concept introduced by considering values of n other than unity. Indeed, it has been claimed that polymerisation of an acceptor may be regarded simply as an extreme form of isomerisation (Koshland and Neet, 1968). However an important difference does exist. Inspection of equation (I-13) reveals that the binding function for an isomerising system is independent of [A] or [C] (and hence of $\bar{c}_A$). On the other hand, the more general equation (I-11) describes specifically a dependence of r on [A], and hence on $\bar{c}_A$. In practical terms, this implies that a polymerising acceptor may give rise to a *family* of distinct binding curves, each curve corresponding to a particular total concentration of

the acceptor. An example of behaviour of this type has been observed in the binding of guanosine triphosphate to the polymerising glutamate dehydrogenase system (Frieden and Colman, 1967). This phenomenon may be examined more closely by considering a dimerising acceptor system with $\beta = 0$, for which equation (I-8) becomes a quadratic polynomial in [A],

$$2M_A X[A]^2 + M_A(1+\alpha)^p[A] - \bar{c}_A = 0; \quad \alpha = K_A[S] \qquad (I-14)$$

The positive root of equation (I-14), substituted in equation (I-11) yields

$$r = \frac{2pM_S\alpha(1+\alpha)^{p-1}}{M_A(1+\alpha)^p + \{M_A^2(1+\alpha)^{2p} + 8M_A X\bar{c}_A\}^{\frac{1}{2}}} \qquad (I-15)$$

This formulation shows that the product $X\bar{c}_A$ is the important quantity in dictating the form of a binding curve for a dimerising system. An illustration with numerical examples is presented in Figure (I-1). The interplay between the concentration of the acceptor and the equilibrium constant X (itself a function of several variables) will be discussed later.

(c) Elaboration of the model for a self-interacting acceptor

When $n > 2$ the improbability of multiple body collisions suggests a pathway for higher polymer formation involving polymers of intermediate size. There is, however, no thermodynamic requirement that the relative amounts of intermediate polymers need be appreciable at equilibrium (Langerman and Klotz, 1969). In the event that a series of polymeric (or isomeric) forms of the acceptor do coexist in

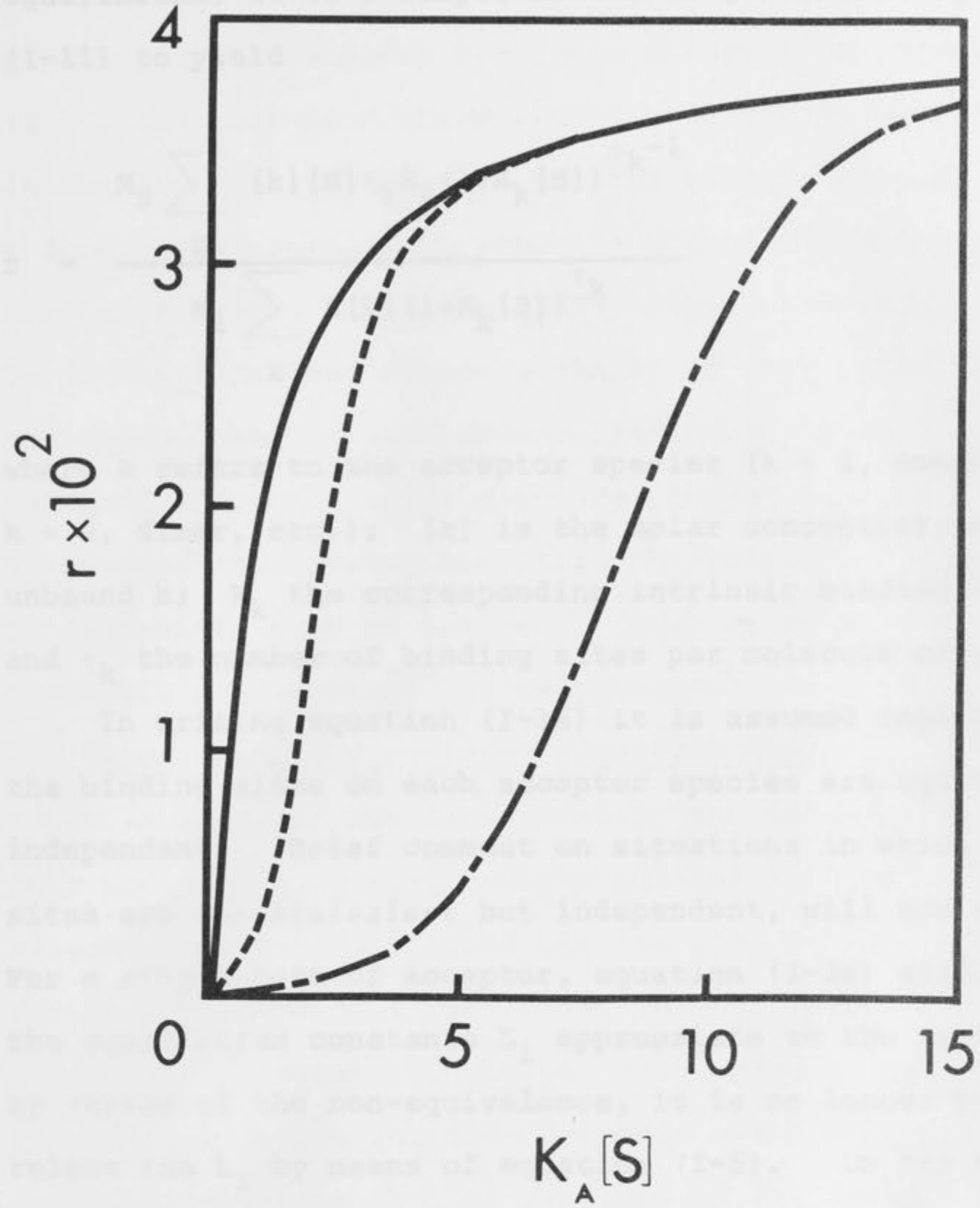


FIGURE I-1

Binding curves computed for a system in which ligand of molecular weight 200 binds to four equivalent and independent sites on the monomeric form of acceptor (M_A = 20,000), which coexists in equilibrium with a non-binding dimer. Values of $X\bar{m}_A$, made dimensionless by expressing total acceptor as base-moles/l (= $\bar{c}_A/M_A$), are as follows: ——, 0.5; ---, 0.5 x 10^4; -·-, 0.5 x 10^8.

equilibrium, it is a simple matter to generalise equation (I-11) to yield

$$r = \frac{M_S \sum_k [k][S]\tau_k K_k (1+K_k[S])^{\tau_k - 1}}{M_1 \sum_k k[k](1+K_k[S])^{\tau_k}} \qquad (I\text{-}16)$$

where k refers to the acceptor species (k = 1, monomer; k = 2, dimer, etc.); [k] is the molar concentration of unbound k; K_k the corresponding intrinsic binding constant, and τ_k the number of binding sites per molecule of species k.

In writing equation (I-16) it is assumed implicitly that the binding sites on each acceptor species are equivalent and independent. Brief comment on situations in which binding sites are *non-equivalent* but independent, will now be given. For a *single* form of acceptor, equation (I-3a) still defines the equilibrium constants L_i appropriate to the system; but, by virtue of the non-equivalence, it is no longer possible to relate the L_i by means of equation (I-5). On the other hand, using the method of partial fractions, it is a simple matter to derive the appropriate binding equation;

$$r_m = \sum_{i=1}^{p} \frac{L_i[S]}{1+L_i[S]} \qquad (I\text{-}17)$$

A plot of r_m *vs.* [S] according to equation (I-17) will deviate from the form of a rectangular hyperbola, but will not be sigmoidal (Blake and Peacocke, 1968). This behaviour may be more easily described by referring to a double reciprocal plot of $1/r_m$ *vs.* 1/[S]. For systems describing

a rectangular hyperbola (equation I-12) the double reciprocal plot is linear, whereas for those described by equation (I-17) the plot is a curve with decreasing slope as 1/[S] increases. In contrast, sigmoidal binding results produce a double reciprocal plot which is convex (exhibiting upward curvature) relative to the 1/[S] axis. The important point is that a sigmoidal response cannot be attributed to the non-equivalence of independent binding sites on a single acceptor species. If a sigmoidal response is obtained with a non self-interacting acceptor, the concept of *dependent* binding sites must be invoked. Such sites have been called 'mutually interacting' or 'cooperative'.

2. Ligand induced conformational changes.

The model of Koshland, Némethy and Filmer (1966) assumes that proteins associated with sigmoidal binding curves are oligomeric, with one ligand binding site on each subunit. The conformation of a particular subunit changes from state A to state B during the process of binding a ligand. The free energy change for this compound event contains major contributions from the interactions of the newly liganded subunit and its immediate neighbours, which may be in either the A or B state. The workers defined a product $K_S K_T$ to describe the equilibrium binding of S to an *isolated* subunit and two interaction constants K_{AB} and K_{BB} which reflect the respective stabilities of the A-B and B-B interactions relative to the A-A interaction between two unliganded subunits. At various stages of partial saturation of the oligomeric protein, any one subunit will clearly experience a variety of interactions with its neighbours (depending on which state each has adopted), and hence will exhibit an

apparent variety of affinities for the ligand. Depending on the magnitudes of K_{AB} and K_{BB}, these interactions may lead to a sigmoidal binding curve, and a double reciprocal plot which is convex relative to the 1/[S] axis (positive cooperativity between binding sites) or they may lead to a double reciprocal plot which is concave relative to the 1/[S] axis (negative cooperativity).

Figure (I-2) permits a schematic comparison of the models proposed by Koshland *et al.* (1966) and Monod *et al.* (1965) for the particular case of a protein consisting of two subunits, each of which may bind one molecule of ligand and exist in either one of two distinct conformations. The species in heavy outline are appropriate to the model of Monod *et al.* and the corresponding binding equation may be obtained directly from equation (I-11) by setting p = q = 2 and n = 1,

$$\bar{Y} = \frac{rM_A}{2M_S} = \frac{(K_A+XK_C)[S] + (K_A^2+XK_C^2)[S]^2}{(1+X) + 2(K_A+XK_C)[S] + (K_A^2+XK_C^2)[S]^2} \qquad \text{(I-18)}$$

where $\bar{Y}$, as defined, is termed a saturation function. The pathway indicated by the shaded species represents the sequential transition mechanism proposed by Koshland *et al.* who expressed their saturation function in the form

$$\bar{Y} = \frac{(K_{AB}K_SK_T)[S] + (K_{AB}K_S^2K_T^2)[S]^2}{1 + (2K_{AB}K_SK_T)[S] + (K_{BB}K_S^2K_T^2)[S]^2} \qquad \text{(I-19)}$$

A comparison of equations (I-18) and (I-19) reveals that they are both a ratio of two polynomials in [S] although the meanings attached to the constant coefficients differ in each

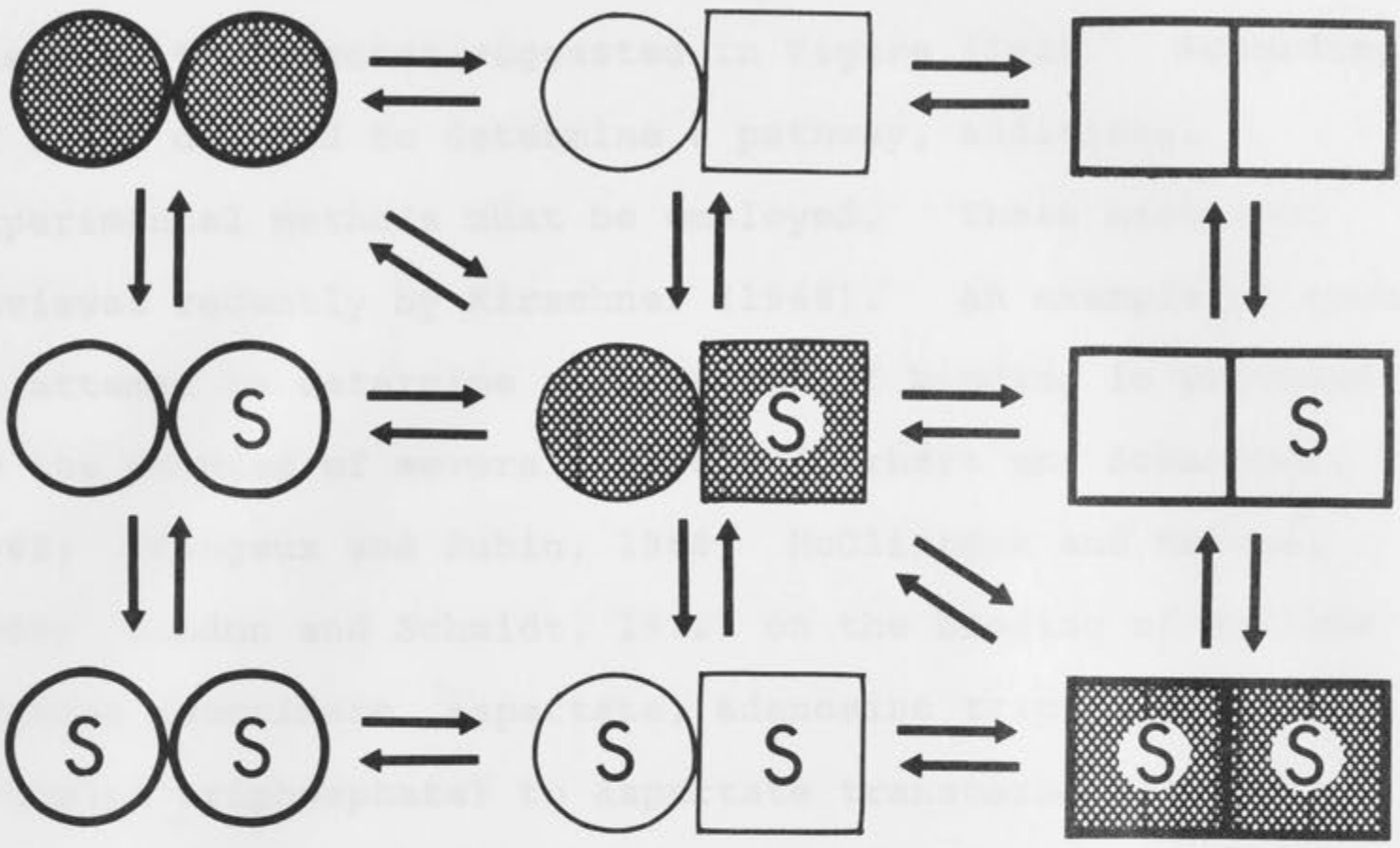


FIGURE I-2

A schematic diagram for the comparison of two models postulated to explain sigmoidal binding. The shaded species are involved in the sequential transition mechanism postulated by Koshland *et al.* (1966) while the species in heavy outline are considered by Monod *et al.* (1965) to coexist in equilibrium.

case. The important point which emerges is that the analysis of thermodynamic binding data obtained with equilibrium mixtures is incapable of distinguishing between the two postulated pathways of complex formation, or indeed the many other pathways which could be conceived on the basis of the species suggested in Figure (I-2). Accordingly, if it is desired to determine a pathway, additional experimental methods must be employed. These have been reviewed recently by Kirschner (1968). An example of such an attempt to determine a mechanism of binding is provided by the studies of several workers (Gerhart and Schachman, 1968; Changeux and Rubin, 1968; McClintock and Markus, 1969; London and Schmidt, 1972) on the binding of various ligands (succinate, aspartate, adenosine triphosphate and cytosine triphosphate) to aspartate transcarbamylase.

SECTION B: DIFFERENTIATION OF BINDING EQUATIONS RELATING TO SELF-INTERACTING ACCEPTORS.

In previous work, the meaning of the word 'sigmoidal' has been ill-defined. In general it is associated with an S-shaped binding curve obtained experimentally or by computation (e.g. Figure I-1). Stadtman (1966) noted that this form of curve offers advantages over a rectangular hyperbola in relation to metabolic control. The amount of ligand bound is relatively small at low ligand concentrations, but rises to a saturating value at higher concentrations. In so doing, the curve exhibits a region in which the amount of ligand bound is a maximally sensitive function of the unbound ligand concentration. It is this feature which appears to be of primary importance (for

example) in the binding and release of oxygen by mammalian haemoglobin, since the red cells must release oxygen to the tissues at a relatively high oxygen tension (Brewer and Eaton, 1971). It would be meaningful, therefore, to define a sigmoidal binding curve as a curve exhibiting a point of inflection at which $d^2r/d[S]^2 = 0$, and $dr/d[S]$ is a maximum. The problem of determining whether binding equations like equations (I-11) and (I-19) describe sigmoidal curves for *all* values of their constants (and there is no *a priori* reason that they should) is now reduced to the problem of determining the conditions for which there exists a real, positive (non zero) value of [S] such that the second derivative equals zero. Although it may be possible to write equation (I-19) more generally, and to perform the required differentiations, the problem is exceedingly difficult, and so attention will be confined, in this thesis, to equation (I-11), describing situations in which the acceptor is either isomerising or polymerising.

1. Isomerising acceptor systems.

(a) The second derivative test

For the sake of easy reference, equation (I-11) is rewritten with n = 1, X = [C]/[A] as

$$r_m = \frac{pK_A[S](1+K_A[S])^{p-1} + qK_CX[S](1+K_C[S])^{q-1}}{(1+K_A[S])^p + X(1+K_C[S])^q} \qquad (I-20)$$

Table (I-1) summarises the conditions under which equation (I-20) describes a curve of the form of a rectangular hyperbola. For conditions other than those listed in the table, the plot of r_m *vs.* [S] deviates from the form of

TABLE I-1

A summary of conditions for which the binding of ligand to an isomerising acceptor system results in a binding curve with the form of a rectangular hyperbola

Condition*	Binding Equation
$p = 1$, q and/or $K_C = 0$	$r_m = K_A[S]/\{1+X+K_A[S]\}$
p and/or $K_A = 0$, $q = 1$	$r_m = K_C X[S]/\{1+X+XK_C[S]\}$
$p = 1$, $q = 1$	$r_m = (K_A+K_C X)[S]/\{(1+X+(K_A+K_C X)[S])\}$
$p = q$, $K_C/K_A = 1$	$r_m = pK_A[S]/\{1+K_A[S]\}$

* p and q are the numbers of binding sites per molecule of isomers, A and C, respectively: K_A and K_C are the respective intrinsic binding constants.

a rectangular hyperbola. The general expression for the second derivative of equation (I-20) is given (after extensive rearrangement) by equation (I-21) which appears on the next page. It is immediately apparent that there is no general explicit expression for [S] corresponding to the point of inflection at which $d^2r/d[S]^2 = 0$. However, it is possible to proceed, provided particular values are assigned to p and/or q, which appear in the exponents. The procedure is illustrated with two examples.

(i) <u>q = 0 with p assuming any integral value greater than unity</u>. With q = 0, equation (I-20) simplifies to the form used by Changeux and Rubin (1968) and Nichol, Smith and Winzor (1969) in their attempts to analyse binding results obtained with the succinate-aspartate transcarbamylase system. The same equation is realised, of course, if K_C is set equal to zero. Equation (I-21) for this case simplifies to

$$(2+pK_A[S])(p-1)X^2 + X(1+K_A[S])^p\{2(p-2)-pK_A[S](1+p)\}$$

$$- 2(1+K_A[S])^{2p} = 0 \qquad \text{(I-22)}$$

when $d^2r/d[S]^2 = 0$. Equation (I-22) may be expanded to yield,

$$2\{(p-1)X-1\}\{X+1\} + \{(p-1)X-4\}\{X+1\}pK_A[S]$$

$$+ \sum_{i=2}^{p} \{\{2(p-2)-ip(p+1)/(p-i+1)\}\binom{p}{i}X-2\binom{2p}{i}\}K_A^i[S]^i$$

$$- \{p(1+p)X+2\binom{2p}{p+1}\}K_A^{p+1}[S]^{p+1} - \sum_{j=p+2}^{2p} 2\binom{2p}{j}K_A^j[S]^j = 0 \qquad \text{(I-23)}$$

Equation (I-21)

$$d^2r/d[S]^2 = H([S])/(x^p+Xy^q)^3$$

$$H([S]) = - 2pK_A^2x^{3p-3} + q(q-1)(2+qK_C[S])XK_C^2x^{2p}y^{q-3}$$

$$- pqXK_AK_Cx^{2p-1}y^{q-2}\{(3q+1)K_C[S]+4\}$$

$$+ 3pq(p+1)XK_A^2K_C[S]x^{2p-2}y^{q-1}$$

$$- pXK_A^2x^{2p-3}y^q\{p(p+1)K_A[S]-2(p-2)\}$$

$$- qX^2K_C^2x^py^{2q-3}\{q(q+1)K_C[S]-2(q-2)\}$$

$$+ 3pq(q+1)X^2K_AK_C^2[S]x^{p-1}y^{2q-2}$$

$$- pqX^2K_AK_Cx^{p-2}y^{2q-1}\{(3p+1)K_A[S]+4\}$$

$$+ p(p-1)(2+pK_A[S])X^2K_A^2y^{2q}x^{p-3}$$

$$- 2qK_C^2X^3y^{3q-3}$$

where $x = 1 + K_A[S]$ and $y = 1 + K_C[S]$

It follows directly that the coefficients of $[S]^2$ and all higher powers of [S] are always negative irrespective of the values of p and X. The constant term in equation (I-23) is positive for all $X > 1/(p-1)$, and an analysis of the coefficient of [S] reveals that it is positive for all $X > 4/(p-1)$. Thus whenever the coefficient of [S] is positive, the constant term is also positive. It is also possible that the coefficient of [S] be negative, and the constant term positive if $1/(p-1) < X < 4/(p-1)$. In either case there is only *one* change of sign in equation (I-23) and by Descartes' Rule of Sign, there is only one positive root, $[S]_c$, which satisfies equation (I-22) and hence defines a single point of inflection in the binding curve. Conversely, when $0 < X < 1/(p-1)$, the binding curve, while deviating from the form of a rectangular hyperbola, exhibits no point of inflection. In summary, the condition that a binding curve be sigmoidal when n = 1 and q = 0 is

$$X > 1/(p-1) \qquad \text{(I-24)}$$

(ii) $p = q$, $\beta = K_C/K_A \neq 1$. This is the case considered by Monod *et al*. (1965) in which numbers of binding sites are preserved during isomerisation but with altered affinities for ligand. The case $\beta = 1$ has been mentioned in Table (I-1). Consider first the example $p = q = 2$, $\beta \neq 1$ for which equation (I-21) becomes

$$- (K_A^2+XK_C^2)^2(K_A+XK_C)[S]^3 - 3(1+X)(K_A^2+XK_C^2)[S]^2$$

$$- 3(1+X)(K_A^2+XK_C^2)(K_A+XK_C)[S] - (1+X)\{K_A^2(1-X)+K_C^2X(X-1)+4K_AK_CX\} = 0$$

(I-25)

when $d^2r/d[S]^2 = 0$. The coefficients of all terms involving [S] are negative and only the sign of the constant term in the polynomial may be varied. By Descartes' Rule, if there is to be a positive root of equation (I-25) and hence a point of inflection in the binding curve, it is required that the constant term be positive. This condition may be written, after division by $K_A^2(1+X)$, as

$$\psi = (1-X) + \beta^2X(X-1) + 4\beta X < 0 \qquad \text{(I-26)}$$

It follows immediately that when $X = 1$, there can be no point of inflection in the binding curve since β cannot be less than zero. However, such a point may exist if $0 < X < 1$ or $X > 1$. In the former case, a plot of ψ *vs.* β exhibits a maximum ($d^2\psi/d\beta^2 < 0$) and intersects the β axis at the points

$$\beta = \frac{\sqrt{X}(\sqrt{X}-1)}{X(\sqrt{X}+1)} \quad \text{and} \quad \frac{-\sqrt{X}(\sqrt{X}+1)}{X(\sqrt{X}-1)} \qquad \text{(I-27)}$$

When $X > 1$, the plot of ψ *vs.* β exhibits a minimum ($d^2\psi/d\beta^2 > 0$) and intersects the β axis at the same points, which now assume opposite sign. The plots are sketched in Figure (I-3a) and (I-3b), the shaded areas denoting the regions conforming to inequality (I-26). The conditions for the existence of a point of inflection in the binding curve may now be summarised as

$$\beta > \frac{-\sqrt{X}(\sqrt{X}+1)}{X(\sqrt{X}-1)} \quad ; \quad 0 < X < 1,\ p = q = 2,\ \beta \neq 1 \qquad \text{(I-28a)}$$

$$0 < \beta < \frac{\sqrt{X}(\sqrt{X}-1)}{X(\sqrt{X}-1)} \quad ; \quad X > 1,\ p = q = 2,\ \beta \neq 1 \qquad \text{(I-28b)}$$

FIGURE I-3

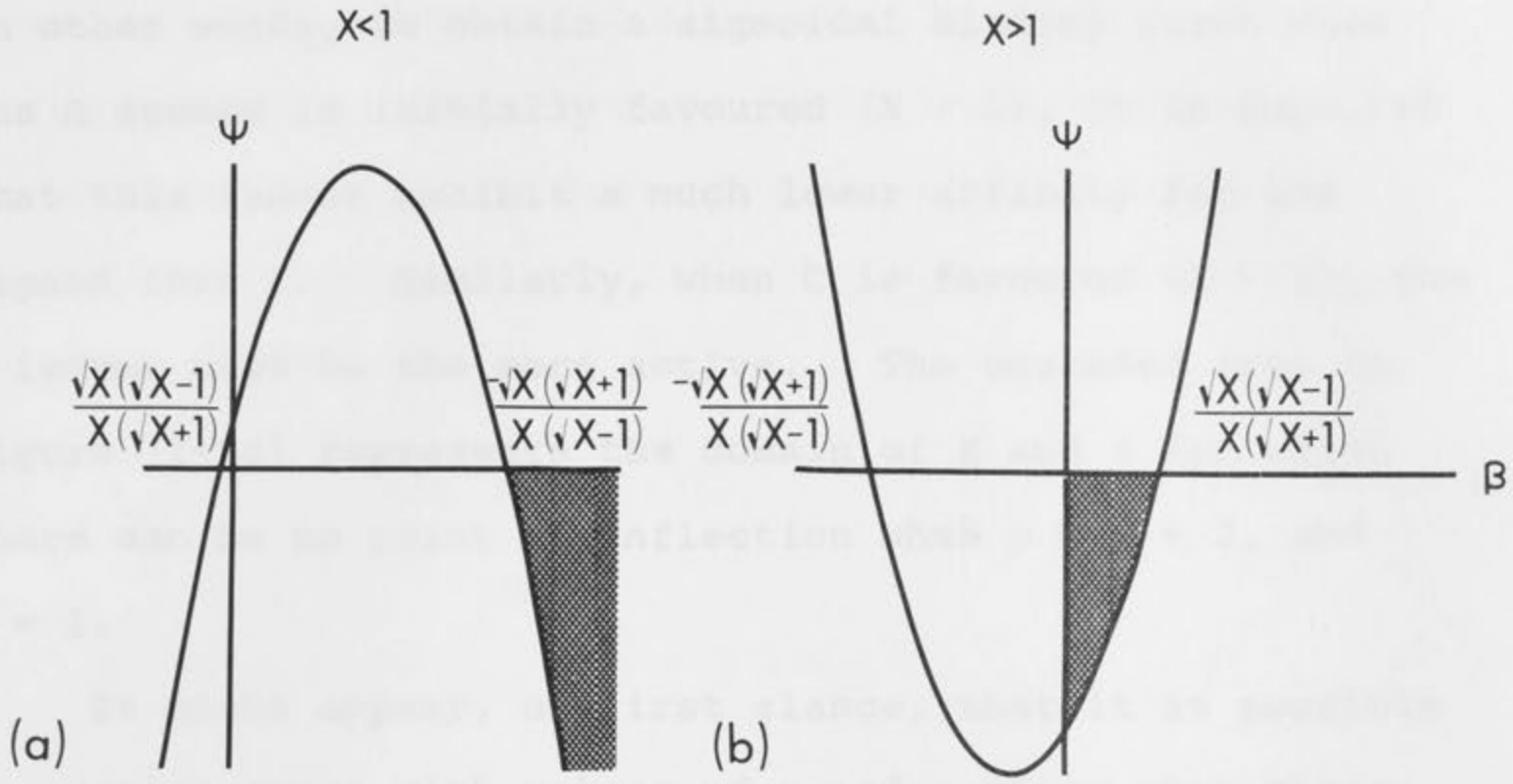
Determination of the domains of sigmoidality for the case involving the binding of a ligand to two sites on each of two isomeric forms of acceptor coexisting in an equilibrium defined by the isomerisation constant X. The ratio of the intrinsic binding constants (relevant to each isomer) is termed β. The condition for the existence of a point of inflection in the binding curve is given by equation (I-26),

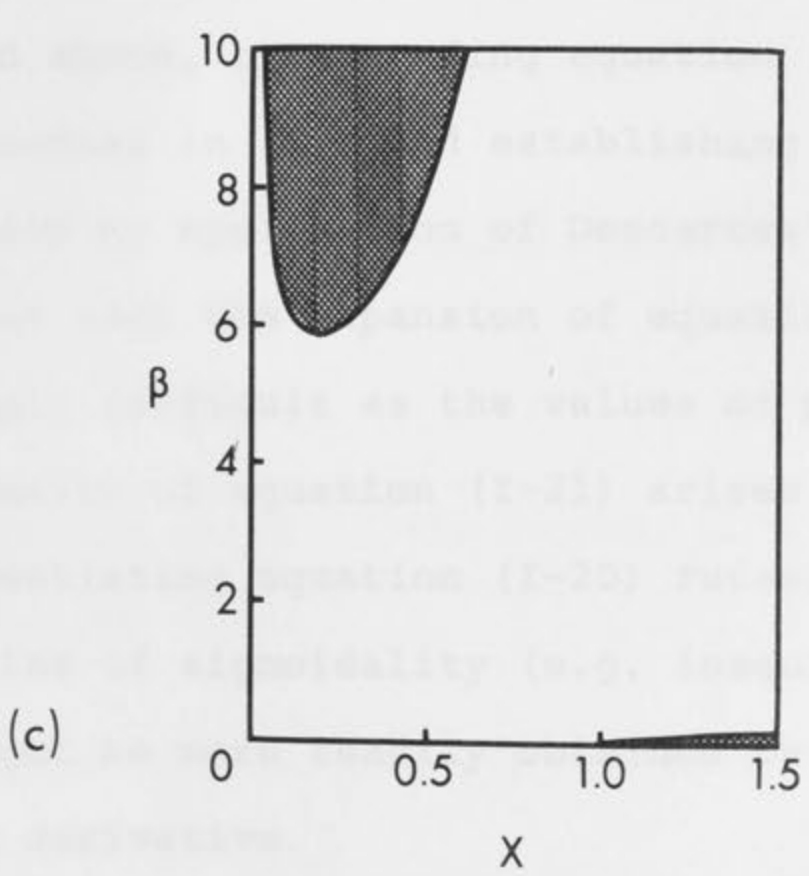
$$\psi = (1-X)+\beta^2 X(X-1)+4\beta X < 0$$

(a) A sketch of ψ *vs.* β for $0 < X < 1$.

(b) A similar sketch for $X > 1$.

(c) Shaded areas denoting the domains in the X,β plane for which the binding curve is sigmoidal.


X<1
X>1
Ψ
Ψ
$\frac{\sqrt{X}(\sqrt{X}-1)}{X(\sqrt{X}+1)}$
$-\frac{\sqrt{X}(\sqrt{X}+1)}{X(\sqrt{X}-1)}$
$-\frac{\sqrt{X}(\sqrt{X}+1)}{X(\sqrt{X}-1)}$
$\frac{\sqrt{X}(\sqrt{X}-1)}{X(\sqrt{X}+1)}$
β
(a)
(b)


10
8
6
4
2
0
β
0.5
1.0
1.5
X
(c)

The shaded areas in Figure (I-3c) show (in part) the two domains of X and β which satisfy inequalities (I-28a) and (I-28b). If the A isomer is initially favoured ($X < 1$) it can be seen that β must assume values well above unity. In other words, to obtain a sigmoidal binding curve when the A isomer is initially favoured ($X < 1$), it is required that this isomer exhibit a much lower affinity for the ligand than C. Similarly, when C is favoured ($X > 1$), the A isomer must be the more active. The unshaded area in Figure (I-3c) represents the domain of X and β for which there can be no point of inflection when $p = q = 2$, and $n = 1$.

It might appear, on first glance, that it is possible to examine cases with values of p and q other than those considered above, by expanding equation (I-21) in the form of a polynomial in [S], and establishing domains of sigmoidality by application of Descartes' Rule. However, it is clear that the expansion of equation (I-21) becomes increasingly difficult as the values of p and q increase. The complexity of equation (I-21) arises as a consequence of differentiating equation (I-20) *twice*, and it was felt that domains of sigmoidality (e.g. inequalities (I-24) and (I-28)) might be more readily obtained by a method based on the first derivative.

(b) The first derivative test

Scatchard (1949) has pointed out the usefulness of a plot of r/[S] *vs.* r in the interpretation of binding results. For example, a system associated with a binding curve of the form of a rectangular hyperbola, such as that

described by equation (I-12), will have a corresponding Scatchard plot which is linear with slope $-K_A$, and intercepts pM_SK_A/M_A. Frieden and Colman (1967), have shown that the Scatchard plot corresponding to experimental sigmoidal binding data is a curve exhibiting a distinct maximum. In the present context, therefore, it is of interest to recast equation (I-20) in the Scatchard format, and to determine under which condition $d(r_m/[S])/dr_m = 0$. Equation (I-20) may be written

$$r_m/[S] = F'([S])/F([S]) \qquad \text{(I-29a)}$$

$$F([S]) = (1+K_A[S])^p + X(1+K_C[S])^q \qquad \text{(I-29b)}$$

where $F'([S])$ denotes the first derivative of $F([S])$ with respect to $[S]$. It follows that

$$d(r_m/[S])/dr_m = d[S]/dr_m \frac{\{F([S])F''([S]) - (F'([S]))^2\}}{(F([S]))^2} \qquad \text{(I-30)}$$

At the turning point in the Scatchard plot, $d(r_m/[S])/dr_m = 0$, which requires that

$$F([S])F''([S]) - \{F'([S])\}^2 = 0 \qquad \text{(I-31)}$$

since $d[S]/dr_m \neq 0$ for $0 < [S] < \infty$. Equation (I-31) may be written as a polynomial in $[S]$

$$- pK_A^2 x^{2p-2} - qX^2K_C^2y^{2q-2} + q(q-1)XK_C^2x^py^{q-2}$$

$$+ p(p-1)XK_A^2x^{p-2}y^q - 2pqXK_AK_Cx^{p-1}y^{q-1} = 0 \qquad \text{(I-32)}$$

where $x = 1 + K_A[S]$ and $y = 1 + K_C[S]$. While equation (I-32) is very much simpler than equation (I-21), it is still necessary to assign particular values to p and q in order to proceed. The question of interest is whether the conditions required for the existence of a turning point in the Scatchard plot are the same as those required for sigmoidality

(i) $\underline{q = 0,\ p > 1}$. For this case equation (I-32) becomes, on division by $pK_A^2x^{p-2}$,

$$-(1+K_A[S])^p + (p-1)X = 0 \qquad \text{(I-33)}$$

which by Descartes' rule of sign may have only one positive real root (corresponding to a single turning point) when $(p-1)X - 1 > 0$, i.e. $X > 1/(p-1)$. This is precisely the condition stipulated by inequality (I-24), and thus for the case $q = 0$ the domain of values of X for which the binding curve exhibits a point of inflection is identical to that for which a turning point (in fact a maximum) is found in the Scatchard plot.

(ii) $\underline{p = q,\ \beta \neq 1}$. The investigation is continued by considering, first, the case $p = q = 2$, for which equation (I-32) reduces to

$$-(X^2K_C^2+K_A^2)[S]^2 - (XK_C^2+K_A^2)(XK_C+K_A)[S]$$

$$-\{K_A^2(1-X) + K_C^2X(X-1) + 4K_AK_CX\} = 0 \qquad \text{(I-34)}$$

It follows that a positive real root exists only if $(1-X) + \beta^2X(X-1) + 4\beta X < 0$, which is recognized as the

condition formulated previously in inequality (I-26). The correspondence between the appearance of a point of inflection in the binding curve and a turning point in the Scatchard plot is noted once more.

In an attempt to generalise this correlation further, it was found that it is a relatively simple matter to obtain *general* expressions for the constant terms of equations (I-21) and (I-32) with p = q. These terms are, respectively,

$$- 2pK_A^2(1+X)\{[1-(p-1)X] + X[X-(p-1)]\beta^2+2pX\beta\}$$

and $$- pK_A^2\{[1-(p-1)X] + X[X-(p-1)]\beta^2+2pX\beta\}$$

The two terms, therefore, change sign in concert. If the coefficients of all terms involving [S] are negative, it follows that the appearance of a point of inflection in the binding curve and of a turning point in the Scatchard plot are dictated by the *same* condition, viz.,

$$[1-(p-1)X] + X[X-(p-1)]\beta^2 + 2pX\beta < 0 \qquad \text{(I-35)}$$

The relationships between X and β which satisfy this inequality are summarised in Table (I-2). However, it is not implied that when p = q > 2 that there is necessarily only a *single* point of inflection in the binding curve or a *single* turning point in the Scatchard plot.

Consider the case p = q = 3 for which equation (I-32) (relating to a Scatchard plot) becomes

TABLE I-2

A summary of conditions satisfying inequality (I-35)

$$X < \frac{1}{p-1} \quad ; \quad \beta > \frac{-pX-(X+1)\sqrt{(p-1)X}}{X(X-(p-1))}$$

$$\frac{1}{(p-1)} \leq X < p-1 \quad ; \quad 0 < \beta < \frac{-pX+(X+1)\sqrt{(p-1)X}}{X(X-(p-1))} \text{ , and}$$

$$\beta > \frac{-pX-(X+1)\sqrt{(p-1)X}}{X(X-(p-1))}$$

$$X = p-1 \quad ; \quad 0 < \beta < \frac{p-2}{2(p-1)}$$

$$X > p-1 \quad ; \quad 0 < \beta < \frac{-pX+(X+1)\sqrt{(p-1)X}}{X(X-(p-1))}$$

$$- (K_A^6+X^2K_C^6+2XK_A^3K_C^3)[S]^4 - 4(K_A^5+X^2K_C^5+XK_A^3K_C^2+XK_A^2K_C^3)[S]^3$$

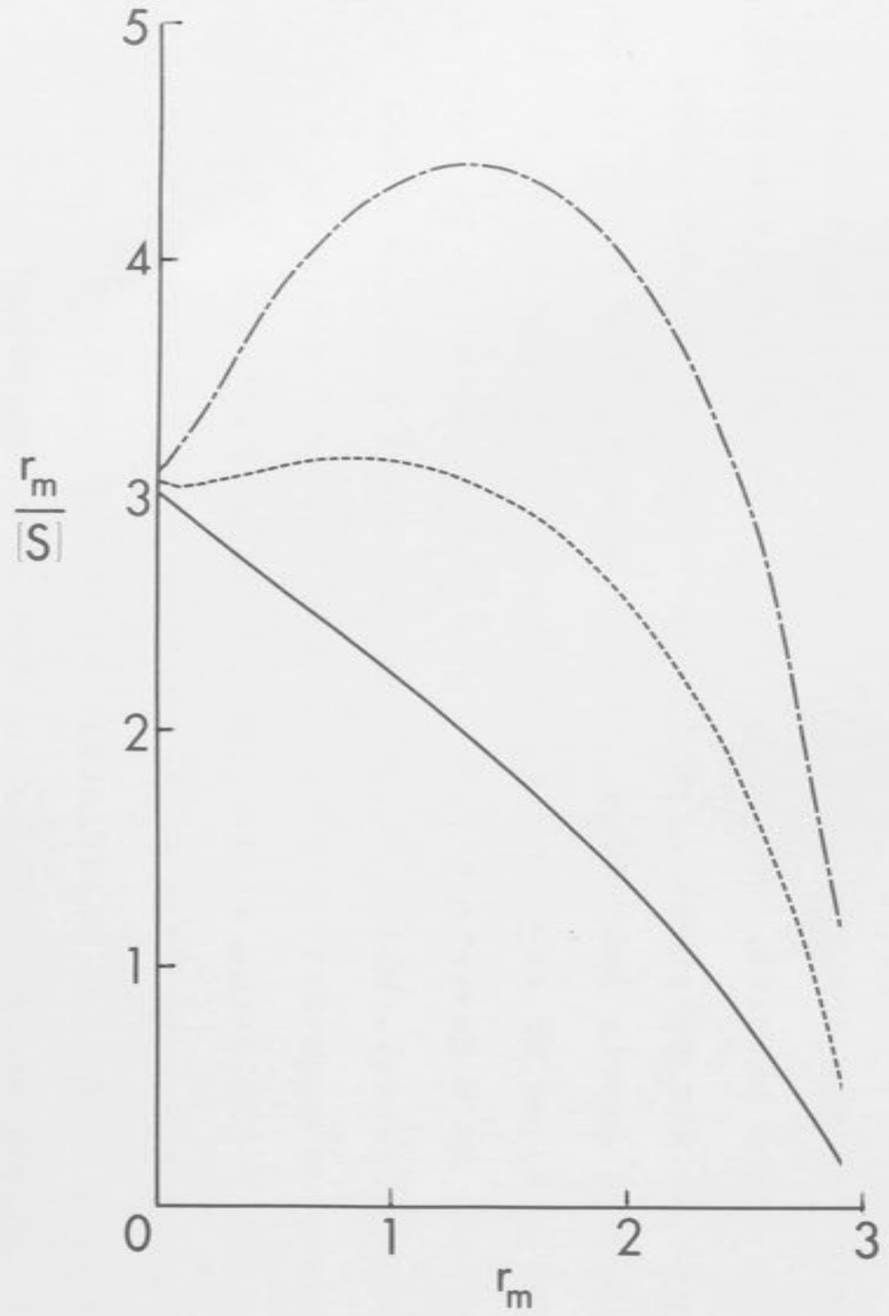
$$- 6(K_A^4+X^2K_C^4+2XK_A^2K_C^2)[S]^2 - 2(2K_A^3+2X^2K_C^3-XK_C^3-XK_A^3+3XK_A^2K_C-6XK_AK_C^2)[S]$$

$$- (K_A^2+X^2K_C^2-2XK_C^2-2XK_A^2+6XK_AK_C) = 0 \qquad \text{(I-36)}$$

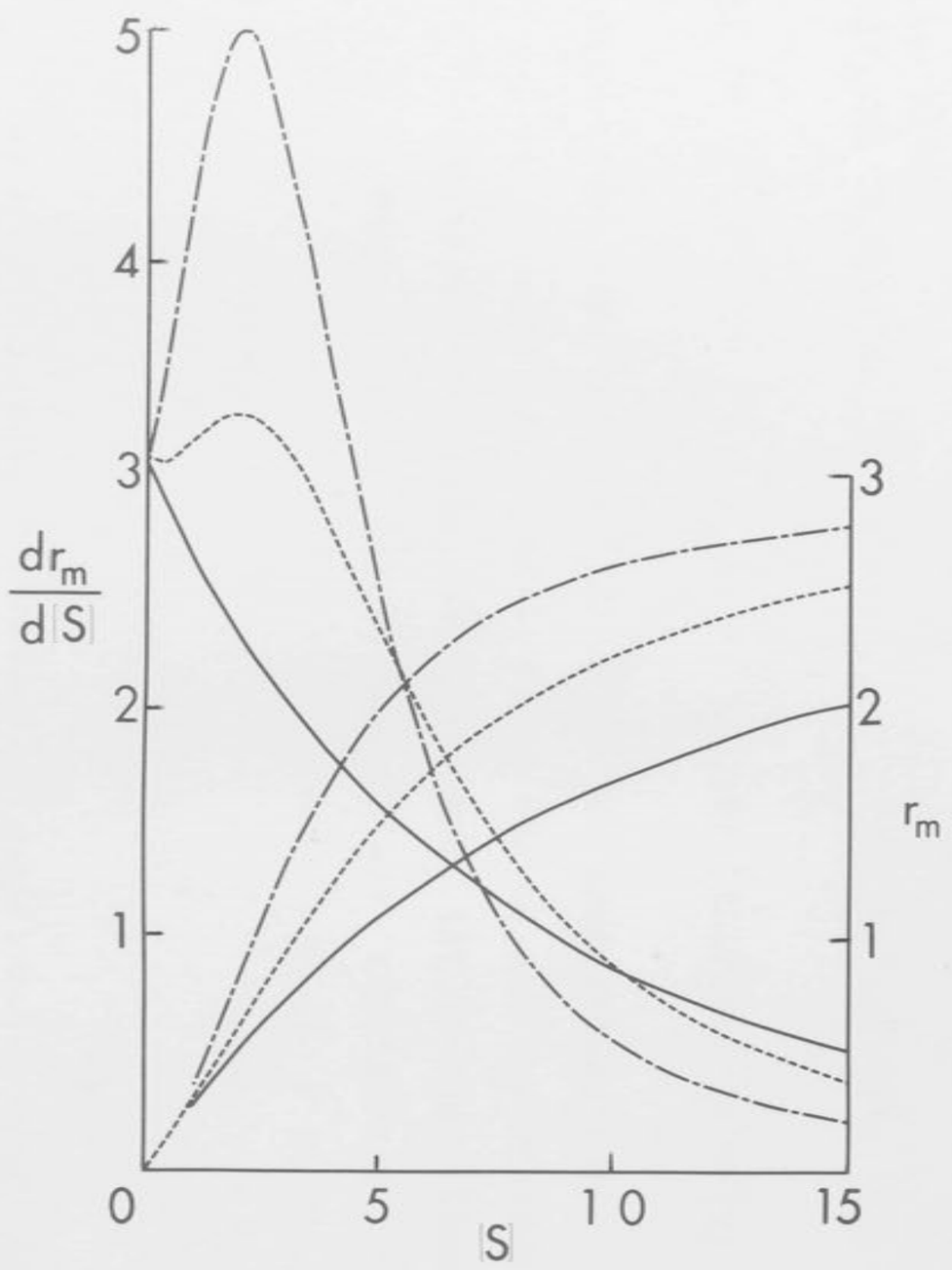
The coefficients of $[S]^4$, $[S]^3$ and $[S]^2$ are necessarily negative but due to the uncertainty in the sign of the coefficient of [S], the following possibilities arise

possibility	coefficient of [S]	constant term
1	-	-
2	-	+
3	+	+
4	+	-

The first possibility, by Descartes' rule, predicts no turning point, while the second implies a single turning point in the domains of X and β specified in Table (I-2). The third possibility specifies, again, only one change of sign in the polynomial, and hence only one turning point, but the fourth introduces the new finding that there may be *two* changes of sign in the polynomial, and hence *two* turning points in a Scatchard plot. An illustration is provided in Figure (I-4a), which shows the Scatchard plots computed, by means of equation (I-29), for the case p = q = 3 and X = 0.001. Since X < 0.5 the first line of Table (I-2) shows that β must be greater than 23.9 if the constant term is to be positive. When β = 30, the coefficient of [S] is positive (possibility 3, above) and the single turning point observed is in accordance with the theory. When β = 10, both the constant term and the coefficient are negative (possibility 1) and,



(a)



(b)

FIGURE I-4

Computed binding curves for a system involving the binding of ligand to three sites on each of two isomeric forms of acceptor coexisting in equilibrium ($X = 1 \times 10^{-3}$): ——, $\beta = 10$; ---, $\beta = 20$; -·-, $\beta = 30$.

(a) Scatchard plots indicating 0, 1 and 2 turning points.

(b) Corresponding binding curves and their first derivatives.

as expected, there is no turning point. In contrast, when $\beta = 20$, the constant term is negative but the coefficient of [S] is positive (possibility 4) and the Scatchard plot contains two turning points (a minimum and a maximum). The corresponding binding curve (Figure 1-4b) contains two points of inflection, and although the point associated with a *minimum* in $dr_m/d[S]$ is difficult to detect, its existence is evident from the first derivative plot of the same curve.

In summary, three points emerge from the examination of the isomerisation model proposed by Monod *et al.* (1965). Firstly, it is not sufficient to stipulate that a ligand must bind preferentially to one isomer, in order to observe a sigmoidal binding curve. The concept is valuable in a qualitative sense, but lacks exact definition. There are clearly regions in the X,β plane for which the binding curve will exhibit no point of inflection, even though the binding might be considered to be preferential. Secondly, methods by which these regions can be defined have been presented, and for all the cases examined, an exact correspondence between the appearance of a point of inflection in the binding curve, and a turning point in the Scatchard plot, was observed. Thirdly, for particular values of p and q, there exist domains of X and β for which more than one point of inflection will appear in the binding curve.

2. Polymerizing acceptor systems.

The basic binding equation for an acceptor undergoing the reaction $nA \rightleftharpoons C$ $(n > 1)$ is equation (I-11). It is clear that r is a function of both [S] and [A] and that the expression for the double derivative $d^2r/d[S]^2$ involves terms in $d[A]/d[S]$ and $d^2[A]/d[S]^2$. While expressions for the

latter derivatives are available from equation (I-8), the final equation for $d^2r/d[S]^2$ contains so many terms that it cannot be examined readily for positive, real roots. In this sense, the second derivative test for sigmoidality fails for a polymerising system, and attention is focussed, therefore, on the first derivative approach. Equation (I-11), written in the Scatchard format becomes

$$r_m/[S] = \{pK_A[A](1+K_A[S])^{p-1} + qK_CX[A]^n(1+K_C[S])^{q-1}\}/\bar{m}_A \qquad (I\text{-}37)$$

where $r_m = M_Ar/M_S$, and $\bar{m}_A(=\bar{c}_A/M_A)$ is the base-molar concentration of all forms of acceptor defined in equation (I-8)

$$\bar{m}_A = [A](1+K_A[S])^p + nX[A]^n(1+K_C[S])^q \qquad (I\text{-}38)$$

Differentiation of equation (I-37) with respect to r_m, and equation (I-38) with respect to [S], leads, on combination of the results, to the following expression when $d(r_m/[S])/dr_m = 0$

$$- pK_A^2[A]x^{2p-2} - 2npqK_AK_CX[A]^nx^{p-1}y^{q-1} + n^2p(p-1)K_A^2X[A]^nx^{p-2}y^q$$

$$+ q(q-1)K_C^2X[A]^nx^py^{q-2} - n^2qX^2K_C^2[A]^{2n-1}y^{2q-2} = 0 \qquad (I\text{-}39)$$

As before, $x = 1+K_A[S]$, $y = 1+K_C[S]$ and it is assumed implicitly that $d[S]/dr_m \neq 0$ for $0 < [S] < \infty$. It can be seen that equation (I-39) is a generalised form of equation (I-32) by substituting $n = 1$ and dividing throughout by [A].

Equation (I-39) will now be used to determine the conditions for which there is a turning point in the

Scatchard plot. For simplicity, discussion is restricted to the general case considered for an isomerising acceptor *viz.* q = 0, p any value. Equation (I-39) simplifies, on division by $pK_A^2[A]x^{p-2}$, to

$$x^p = (1+K_A[S]_c)^p = n^2(p-1)X[A]_c^{n-1} \qquad \text{(I-40)}$$

where $[S]_c$ and $[A]_c$ are the particular values of [S] and [A] corresponding to a turning point in the Scatchard plot, but which, nevertheless must satisfy equation (I-38). It follows from equations (I-38) and (I-40) that

$$[A]_c = \{\bar{m}_A/nX(n(p-1)+1)\}^{1/n} \qquad \text{(I-41a)}$$

and $$[S]_c = \{\{n^2(p-1)X[A]_c^{n-1}\}^{1/p}-1\}/K_A \qquad \text{(I-41b)}$$

which is an acceptable solution provided $[S]_c > 0$ and $0 < [A]_c < \bar{m}_A$. From equations (I-41a and b) it can be shown that the condition $[S]_c > 0$ requires that

$$X\bar{m}_A^{n-1} > \{(n^2(p-1)+n)/n^2(p-1)\}^n/\{n^2(p-1)+n\} \qquad \text{(I-42a)}$$

and the condition $0 < [A]_c < \bar{m}_A$ requires that

$$X\bar{m}_A^{n-1} > 1/\{n^2(p-1)+n\} \qquad \text{(I-42b)}$$

Obviously when inequality (I-42a) is satisfied, inequality (I-42b) must also be satisfied, and subsequent discussion need only be concerned with the former.

Firstly, it can be seen that inequality (I-42a) is a generalised form of inequality (I-24) by setting n = 1. Neither inequality contains a term in β, which is to be

expected, since both models involve a non-binding isomer or polymer. Nor is it surprising that inequality (I-42a) contains a term in $\bar{m}_A$ while inequality (I-24) does not, since only the binding curves for polymerising acceptors are dependent on the concentration of the acceptor. This latter point will be further illustrated by considering a dimerisation (n = 2), for which inequality (I-42a) becomes

$$X\bar{m}_A > (2p-1)/8(p-1)^2 \qquad \text{(I-43)}$$

If p > 1 a turning point in the Scatchard plot is always possible, provided $X\bar{m}_A$ exceeds a number whose value decreases as p increases. In Figure (I-1) it was shown that the value of the product $X\bar{m}_A$ was important in determining the extent of sigmoidality. It now appears that this value is critical in determining whether a binding curve will be sigmoidal* at all. When p = 1 inequality (I-43) can never be satisfied, which does not imply that the binding is unaffected by the dimerisation (in fact it is reduced, to an extent determined by the magnitude of X, over the entire range of [S]) but, simply, that the binding curve can never be sigmoidal.

3. Factors which determine and affect the sigmoidal response.

(a) The total concentration of a polymerising acceptor

Equation (I-15) and inequality (I-42a) have already been used to demonstrate the importance of the product $X\bar{m}_A^{n-1}$ (n > 1) in determining the existence and extent of sigmoidality. It seems reasonable to ask whether the form of the binding curve associated with a polymerising acceptor can be significantly altered by a physically acceptable variation

FOOTNOTE

---

* While it has not been proved generally that domains of sigmoidality are identical to domains for the occurrence of a turning point in a Scatchard plot, the existence of such a proof has been assumed in the remainder of the text. The assumption appears reasonable in view of the number of particular cases for which proof has already been given. Furthermore, it has been found that the computed binding curve exhibits a point of inflection if, and only if, the corresponding Scatchard plot contains a turning point. It must be pointed out that in general, the value of [S] corresponding to a point of inflection is not the same value at the turning point ($r_m/[S]$, r_m) in the Scatchard plot.

---

in $\bar{m}_A$, alone. Although the question is subjective, it does seem unreasonable to propose, for example, that the curves presented in Figure (I-1) could be obtained at fixed X, by varying $\bar{m}_A$ over a 10^8 fold concentration range. However, Figure (I-1) refers only to a dimerising system (n = 2) with 4 binding sites on A and the relevant product is $X\bar{m}_A$. For higher values of n, much smaller variations in $\bar{m}_A$ are required to produce a large change in $X\bar{m}_A^{n-1}$. Figure (I-5) presents computed binding curves for a system in which a monomer possessing six binding sites polymerises to form an inactive hexamer. The values of the parameters reported in the caption, are those found by Nichol, Smith and Winzor (1969) to describe the binding of guanosine triphosphate to glutamate dehydrogenase (Frieden and Colman, 1967). Inequality (I-42a) is directly applicable to this system and predicts that sigmoidality will occur only if $X\bar{m}_A^5 > 0.007$. The solid curve in Figure (I-5) corresponds to a value of 0.003 for $X\bar{m}_A^5$, a value lying outside the specified range; and there is no point of inflection. A 25-fold increase increase in $\bar{m}_A$ yields $X\bar{m}_A^5 = 3 \times 10^4$, a value well inside the predicted domain of sigmoidality, and giving rise to a distinct point of inflection in the corresponding binding curve (---). The remaining curve (-·-) in Figure (I-5) corresponds to another 10-fold increase in $\bar{m}_A$, and illustrates, further, the marked effect which the concentration of the acceptor has on the form of the binding curve. It is of considerable interest that several enzymes have been studied over a 100-fold concentration range, and that their specific activities have exhibited a marked

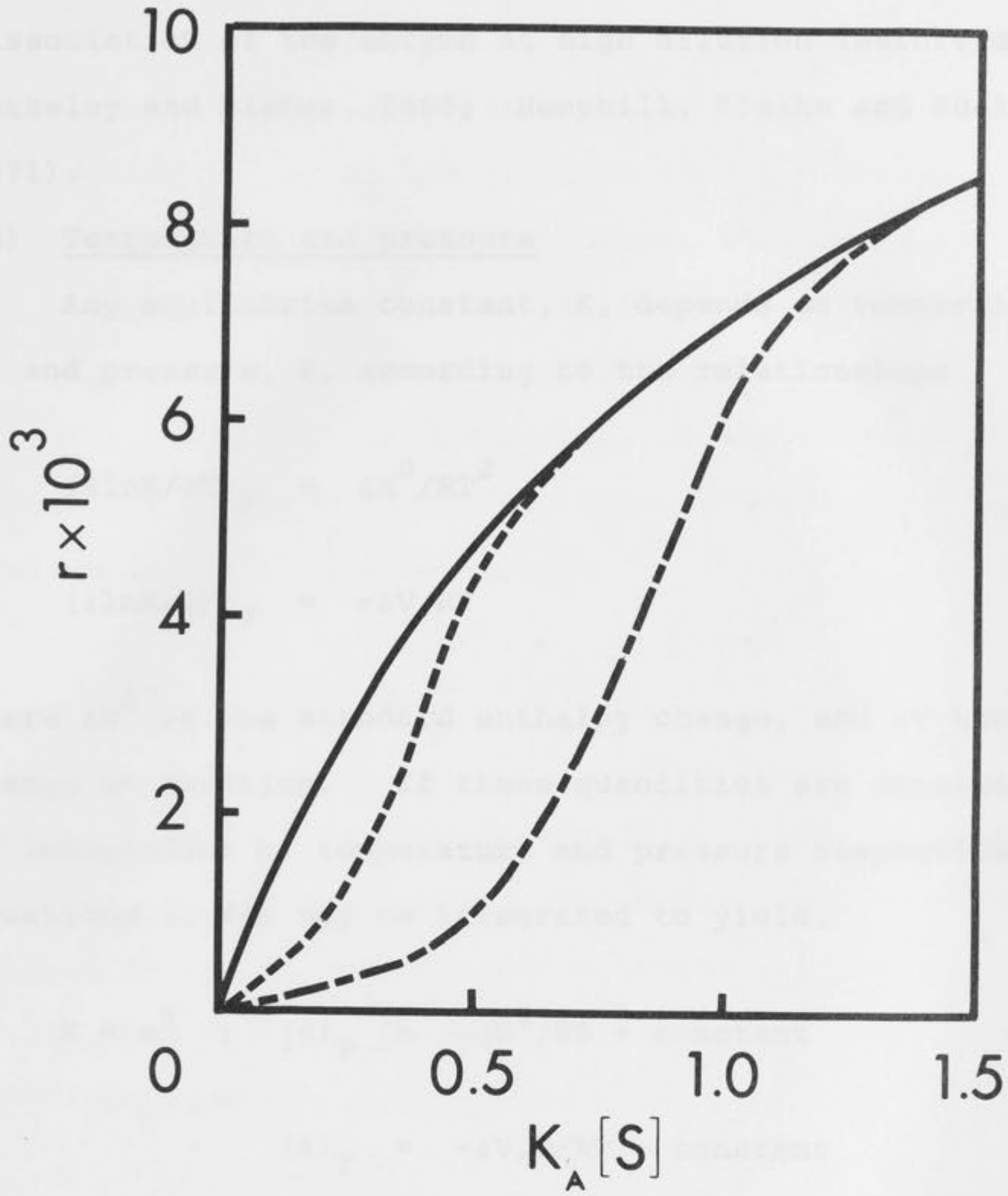


FIGURE I-5

Computed binding curves for a system in which a monomer of molecular weight 300,000, posesses six binding sites (p = 6) and polymerises to form an inactive hexamer (n = 6, q and/or K_C = 0). The intrinsic binding constant K_A and the association constant for hexamer formation, X, were taken as 5×10^6 M^{-1} and 1×10^{36} M^{-5}, respectively. The acceptor concentrations, $\bar{m}_A$ base-moles/l, are as follows ——, 1.33×10^{-8}; ---, 3.33×10^{-7}; -·-, 3.33×10^{-6}.

concentration dependence, which has been attributed to dissociation of the enzyme at high dilution (Bernfeld, Berkeley and Bieber, 1965; Hemphill, Zielke and Suelter, 1971).

(b) Temperature and pressure

Any equilibrium constant, K, depends on temperature, T, and pressure, P, according to the relationships

$$(\partial \ln K/\partial T)_P = \Delta H^0/RT^2 \qquad \text{(I-44a)}$$

$$(\partial \ln K/\partial P)_T = -\Delta V/RT \qquad \text{(I-44b)}$$

where ΔH^0 is the standard enthalpy change, and ΔV the volume change on reaction. If these quantities are considered to be independent of temperature and pressure respectively, equations (I-44) may be integrated to yield,

$$K = e^{\theta} \;;\; (\theta)_P = -\Delta H^0/RT + \text{constant}$$
$$(\theta)_T = -\Delta V.P/RT + \text{constant} \qquad \text{(I-45)}$$

Since any binding equation, or condition for the existence of sigmoidality involves at least one equilibrium constant, temperature and pressure are factors which may determine and govern the form of the sigmoidal response. In the following discussion, it will be assumed that variations in these factors are not sufficient to affect the numbers of binding sites on a particular acceptor species.

(i) The rectangular hyperbola. Equations (I-12) and (I-45) may be combined to give

$$r_m = pe^{\theta}[S]/(1+e^{\theta}[S]) \qquad \text{(I-46)}$$

where, for example, at constant pressure $\theta = -\Delta H_A^0/RT$ + constant, ΔH_A^0 being the standard enthalpy change associated with the intrinsic binding constant K_A. ΔH_A^0 is not directly measurable, but may be related to the total enthalpy change for the binding process as follows. If ΔH_i^0 $(i = 1,2,\ldots,p)$ is defined as the standard enthalpy change for the i-th successive equilibrium $AS_{i-1} + S \rightleftharpoons AS_i$, application of Hess's Law yields

$$(\partial \ln \Pi_i L_i / \partial T)_P = \sum_i \Delta H_i^0/RT^2 \qquad \text{(I-47)}$$

where L_i (defined in equation (I-3a)) is the equilibrium constant for the i-th successive equilibrium. It is related to the intrinsic binding constant K_A by equation (I-5), from which it follows that $\Pi L_i = K_A^p$. Substituting this into equation (I-47) shows that

$$(\partial \ln K_A / \partial T)_P = \sum_i \Delta H_i^0/pRT^2 = \Delta H_A^0/RT^2 \qquad \text{(I-48)}$$

$$\Delta H_A^0 = \sum_i \Delta H_i^0/P \qquad \text{(I-49)}$$

A similar treatment reveals that for a pressure variation at constant temperature, ΔV_A is obtained by dividing the total volume change by the number of equivalent and independent binding sites.

A single expression relating r_m to the temperature, the intrinsic binding constant at any particular temperature,

and the composition ($\bar{m}_A$ and $\bar{m}_S$) of the mixture may be obtained by combining equations (I-1) and (I-46), viz.,

$$e^{\theta}\bar{m}_A r_m^2 - (1+e^{\theta}\bar{m}_S+pe^{\theta}\bar{m}_A)r_m + pe^{\theta}\bar{m}_S = 0 \qquad \text{(I-50)}$$

At constant pressure equation (I-50) may be partially differentiated to yield

$$(\partial r_m/\partial T)_{\bar{m}_S,\bar{m}_A,P} = \frac{e^{\theta}(\Delta H_A^0/RT^2)(\bar{m}_S-r_m\bar{m}_A)(r_m-p)}{e^{\theta}(2r_m\bar{m}_A-p\bar{m}_A-\bar{m}_S)-1} \qquad \text{(I-51)}$$

It follows from equation (I-48) that in a solution of defined composition (i.e. fixed $\bar{m}_S$ and $\bar{m}_A$) the amount of ligand bound increases with increasing temperature when $\Delta H_A^0 > 0$, and decreases with increasing temperature when $\Delta H_A^0 < 0$. Equation (I-51) shows that once a value of K_A has been obtained by constructing a complete binding curve at one temperature, ΔH_A^0 may be found by studying the variation of r_m with T in a *single* mixture, rather than by considering a whole series of complete binding curves at different temperatures. It is also of interest that the variation of r_m with T (or P) depends on the relative values of $\bar{m}_S$ and $\bar{m}_A$: indeed it can be shown that $(\partial r_m/\partial T)_P$ is maximal when $\bar{m}_S = p\bar{m}_A + (1+e^{\theta})$.

(ii) Sigmoidal binding curves. The conditions which define the domains of sigmoidality for both isomerising and polymerising acceptors with q = 0, involve X, but not K_A (inequalities (I-24) and (I-42a)). For these systems, therefore, the enthalpy change of self-interaction, ΔH_X, (or the volume change, ΔV_X) assumes sole importance in

determining the effect of temperature (or pressure) on the shape of the binding curve plotted with $K_A[S]$ as abscissa. The self-interaction constant X_1 at temperature T_1 is related to the constant X_2 at temperature T_2 according to the integrated expression

$$X_1 = X_2 e^{\Delta H_X^0 (1/T_2 - 1/T_1)/R} \qquad \text{(I-52)}$$

For example, a temperature change from 5° to 35° with ΔH_X^0 = 52 Kcals/mole changes X by a factor of 10^4 ($X_1 = 10^4 . X_2$). An isomerising acceptor, with q = 0, exhibits sigmoidal binding when $X > 1/(p-1)$ (inequality (I-24)). Thus it is clear that such a system could be outside this domain at one temperature, and be well inside it at another temperature. Monod *et al.* (1965), in their Figure 1(a), contrast binding curves for this system with X = 1 and 10^4. Both are inside the domain (p = 4, $X > 0.33$), the former curve only marginally, while the latter is pronouncedly sigmoidal. Another example is provided by the binding curves, shown in Figure (I-1) for a dimerising acceptor with p = 2, q = 0. It was pointed out, earlier, that a transition from the solid curve, lying just within the domain of sigmoidality to the broken curve lying well within, was unlikely (in a physiological context) to be due to a 10^4-fold change in $\bar{m}_A$ at fixed temperature and pressure. It can now be seen that such a transition is quite feasible, at fixed $\bar{m}_A$, with a temperature variation from 5° to 35°.

The numbers chosen for the above illustrations are, of course, arbitrary, but they do show that a reasonable variation in temperature may alter significantly the

control properties of certain acceptor-ligand systems. Accordingly, it seems unwise to attribute biological significance to binding (or kinetic) results obtained under conditions which are not physiological. It is interesting to note that Kirschner, Eigen, Bittman and Voigt (1966) have observed that the binding of nicotinamide adenine dinucleotide to the isomerising acceptor D-glyceraldehyde-3-phosphate dehydrogenase describes a rectangular hyperbola at 20° and is sigmoidal at 40°.

When $q \neq 0$ and $\beta = K_C/K_A \neq 0$, a discussion of the effects of temperature on binding to self-interacting acceptors must consider the interplay between β and X in determining whether the response is sigmoidal or not, e.g. equations (I-28a) and (I-28b). The value of $(\beta)_{T_1}$ at temperature T_1 is related to the value $(\beta)_{T_2}$ at T_2 according to

$$\beta_{T_1} = \beta_{T_2} e^{(1/T_2 - 1/T_1)(\Delta H_C^0 - \Delta H_A^0)/R} \qquad \text{(I-53)}$$

Since $(\Delta H_C^0 - \Delta H_A^0)$ is not likely to be large, β is probably relatively insensitive to temperature. For the extreme case in which the difference between the binding affinities of A and C is solely due to an entropic effect (i.e. $\Delta H_C^0 - \Delta H_A^0 = 0$), the locus of (X,β) for varying temperature is a straight line parallel to the X axis. For the particular case considered in Figure (I-3c) it can be seen that such a locus may cross the boundaries of the domains of sigmoidality. Once inside a domain, the variation in the form of the sigmoidal curve with temperature will be

determined primarily by the magnitude and sign of ΔH_X. If q and/or $K_C = 0$, β will be temperature insensitive, but a variation of K_A with temperature ($\Delta H_A \neq 0$) will be reflected in a change of scale of the abscissa axis plotted as $[S] = \alpha/K_A$.

(c) Other factors

Aune and Timasheff (1971) have pointed out that at constant temperature and pressure, any equilibrium constant K, may be regarded as a function of the solvent variables, a_i, where a_i is the activity of species i, which may be a proton, a buffer salt or the solvent itself. Certainly, the apparent equilibrium constants for several protein polymerisations exhibit a dependence on pH and ionic strength (Nichol, Bethune, Kegeles and Hess, 1964), and binding constants may also exhibit such dependencies. The effect of solvent type on protein polymerisations will be discussed in the next Chapter. *In vivo* variations in pH and ionic strength are quite possible in the vicinity of membranes (Goldman *et al.*, 1965), and, by virtue of their effects on self-interaction and binding constants, may dictate whether an acceptor system lies within a domain of sigmoidality or without.

A particular situation which merits more detailed comment at this stage involves the binding of dissimilar ligand molecules to an acceptor, because this introduces the biologically important concept of an effector or modifier. Several workers (Monod *et al.*, 1965; Rubin and Changeux, 1966; Frieden, 1967; Changeux and Rubin, 1968) have examined the action of effectors on isomerising acceptor systems. The present author, in collaboration

with K. O'Dea and L. W. Nichol, has presented a more general treatment (Nichol *et al.*, 1972b). Since the latter work appears as a bound paper at the end of this thesis, only the relevant results are summarised here. Equation (8) of Nichol *et al.* (1972b) may be written as

$$r = \frac{pM_S K_A [S](1+K_A[S])^{p-1} + qM_S K_C X^* [A]^{n-1}[S](1+K_C[S])^{q-1}}{M_A(1+K_A[S])^p + nM_A X^*[A]^{n-1}(1+K_C[S])^q} \qquad \text{(I-54a)}$$

$$X^* = X(1+N_C[E])^y/(1+N_A[E])^{nw} \qquad \text{(I-54b)}$$

where N_A is the intrinsic binding constant referring to the binding of the effector, E, to w equivalent and independent binding sites on A, and N_C is similarly defined in relation to the y sites per molecule of C. Equation (I-54a) is exactly the same as equation (I-11), with X replaced by X*, which, according to equation (I-54b) is a function of the concentration of unbound effector [E], except when $N_A = N_C$ and $y = nw$. When E binds preferentially to C, X* is greater than X, and increases as the total effector concentration increases. In the light of previous discussion it becomes clear that a variation of $\bar{c}_E$ may take a given system from within a domain of sigmoidality to without (or vice versa) depending on which form of acceptor, A or C binds S preferentially. In Chapter IV, the discussion of the action of effectors on a protein polymerisation will be continued with reference to haemoglobin.

4. General Summary.

Equation (I-11), describing the binding of a ligand S to an isomerising (Monod *et al.*, 1965) or polymerising (Nichol *et al.*, 1967a) acceptor, has been used to discuss the sigmoidality of binding curves. For the purposes of this discussion, a binding curve exhibiting a point of inflection was defined as sigmoidal. It has been shown that preferential binding of the ligand to one form of an acceptor is a necessary, but not sufficiently precise condition to ensure sigmoidality which is characterised by the occurrence of a point of inflection in the binding curve. The parameters involved in determining sigmoidality are the numbers of binding sites, p and q, the self-interaction constant X, and the ratio, β, of the affinity constants K_A and K_C. For a polymerising acceptor ($n' > 1$) the total molar concentration of the acceptor, $\bar{m}_A$, is also a determining factor. The domains of these parameters for which the binding curve is sigmoidal have been explicitly defined for a number of cases (inequalities I-24, I-28, I-35, I-42a) and the existence of domains for which the binding curve will exhibit multiple points of inflection has also been revealed. These domains were shown to be identical to those defining the existence of one (or more) turning points in the Scatchard plot.

Variations of the parameters listed above may effect a transition from within a domain of sigmoidality to without. The effect of varying $\bar{m}_A$ is shown in Figure (I-5). It was pointed out that a realistic variation of temperature alone (or pressure) might so alter X as to achieve a pronounced transition. The corresponding effect on β is

smaller. This discussion might also be relevant, in some instances, to kinetic plots of initial velocity *vs.* substrate concentration. Indeed, Frieden (1967) and Dalziel (1968) have shown the formal similarity of equations (I-12) and (I-11) to kinetic equations based on the same models, and on the assumption that the breakdown of enzyme-substrate complexes is rate-limiting. These findings highlight the importance of evaluating the self-interaction constant X, and its dependence on variables such as temperature, pH, ionic strength, and, in a slightly different sense, effector concentration.

CHAPTER II

THE USE OF MASS MIGRATION TECHNIQUES IN EVALUATING POLYMERISATION EQUILIBRIUM CONSTANTS: STUDIES WITH BOVINE β-LACTOGLOBULIN A

In the last Chapter, it was pointed out that the interpretation of binding results obtained with a polymerising acceptor required an evaluation of the polymerisation equilibrium constant(s). Two basic approaches have been employed for this purpose. The first uses the equilibrium methods of osmotic pressure (Kupke, 1960), light scattering (Tanford, 1961) and sedimentation equilibrium (Gibbs, 1928; Fujita, 1962; Creeth and Pain, 1967). These methods are potentially capable of yielding values of successive equilibrium constants when a series of polymers is encountered (Steiner, 1952), and of providing additional thermodynamic information on non-ideality effects (Adams, 1967) and enthalpy changes (Jeffrey and Coates, 1966). The method of sedimentation equilibrium offers two further advantages. Firstly, a range of concentrations may be examined within a single experiment, and secondly, the volume change on polymerisation may be estimated (Howlett, Jeffrey and Nichol, 1970, 1972). This equilibrium method will be discussed in more detail in Chapter IV. The second approach employs the mass migration procedures of sedimentation velocity, moving boundary electrophoresis and frontal gel chromatography (Gilbert, 1955, 1959; Nichol and Winzor, 1972). These methods are characterised by the existence of a plateau region of original composition at all times within the experiment. This is accomplished in chromatography experiments, by loading sufficient solution on to the column to ensure the existence of such a plateau in the elution profile (Winzor and Scheraga, 1963). This Chapter commences with an outline of those sections of the theory of mass migration which are relevant to the present

work. Later, the advantages and limitations of mass migration techniques are discussed with reference to studies on the polymerisation of β-lactoglobulin A, and its dependence on environmental factors.

1. A review of the theory of the mass migration of rapidly polymerising systems.

(a) The conservation of mass condition

In the processes of sedimentation velocity (Svedberg and Pedersen, 1940) and moving boundary electrophoresis (Longsworth, 1959) there is a flow of matter under the influence of an applied potential gradient. Although it is of a quite different nature in each process, the gradient imparts to each solute species a velocity v, which is formulated as a sedimentation coefficient or an electrophoretic mobility. In a frontal chromatographic experiment of the design suggested by Winzor and Scheraga (1963) for which only an elution profile (concentration *vs.* elution volume) is available (*cf.* Brumbaugh and Ackers, 1968), the direct analogue of velocity is the elution volume (Gilbert, 1966a; Nichol, Ogston and Winzor, 1967). If it can be assumed that the cell (or column) in which mass migration occurs has uniform cross-sectional area, and the potential field is homogeneous (the 'rectangular approximation'), then it is possible to derive a single general expression, in terms of the velocity v, for the conservation of mass in a lamina perpendicular to the direction of migration. For the sake of simplicity, it is convenient, though not obligatory, to make the additional assumption that diffusional spreading, being a second order effect, may be neglected for the purposes of determining the

basic features of migration patterns. The theoretical patterns predicted, therefore, are asymptotic solutions at infinite time (Gilbert, 1959). The basic continuity equation expressing conservation of mass is derived first for a single non-self-interacting solute and then extended to a form applicable to a single self-interacting solute.

Consider a single solute moving axially along a tube of uniform cross sectional area S (see Figure II-1). The concentration $c(x,t)$ and velocity $v(x,t)$ of the solute are functions of x, the distance from an arbitrary point on the axis of the tube, and time, t. The net flux of solute in the time interval $(t,t+dt)$ in the lamina bounded by the planes perpendicular to the axis of the tube at x and $x+dx$ is given by

$$Sv(x,t)c(x,t)dt - Sv(x+dx,t)c(x+dx,t)dt$$

and the concomitant change in concentration inside the lamina is

$$\{c(x,t+dt) - c(x,t)\}dV$$

where $dV(= Sdx)$ is the volume of the lamina. If mass is conserved these quantities must be equal, and therefore

$$\{c(x,t+dt)-c(x,t)\}/dt = -\{v(x+dx,t)c(x+dx,t)-v(x,t)c(x,t)\}/dx \qquad \text{(II-1)}$$

In the limit (if it exists) as $dx,dt \to 0$, equation (II-1) may be written

$$\partial c/\partial t = -\partial vc/\partial x \qquad \text{(II-2)}$$

and if v is a function of c only

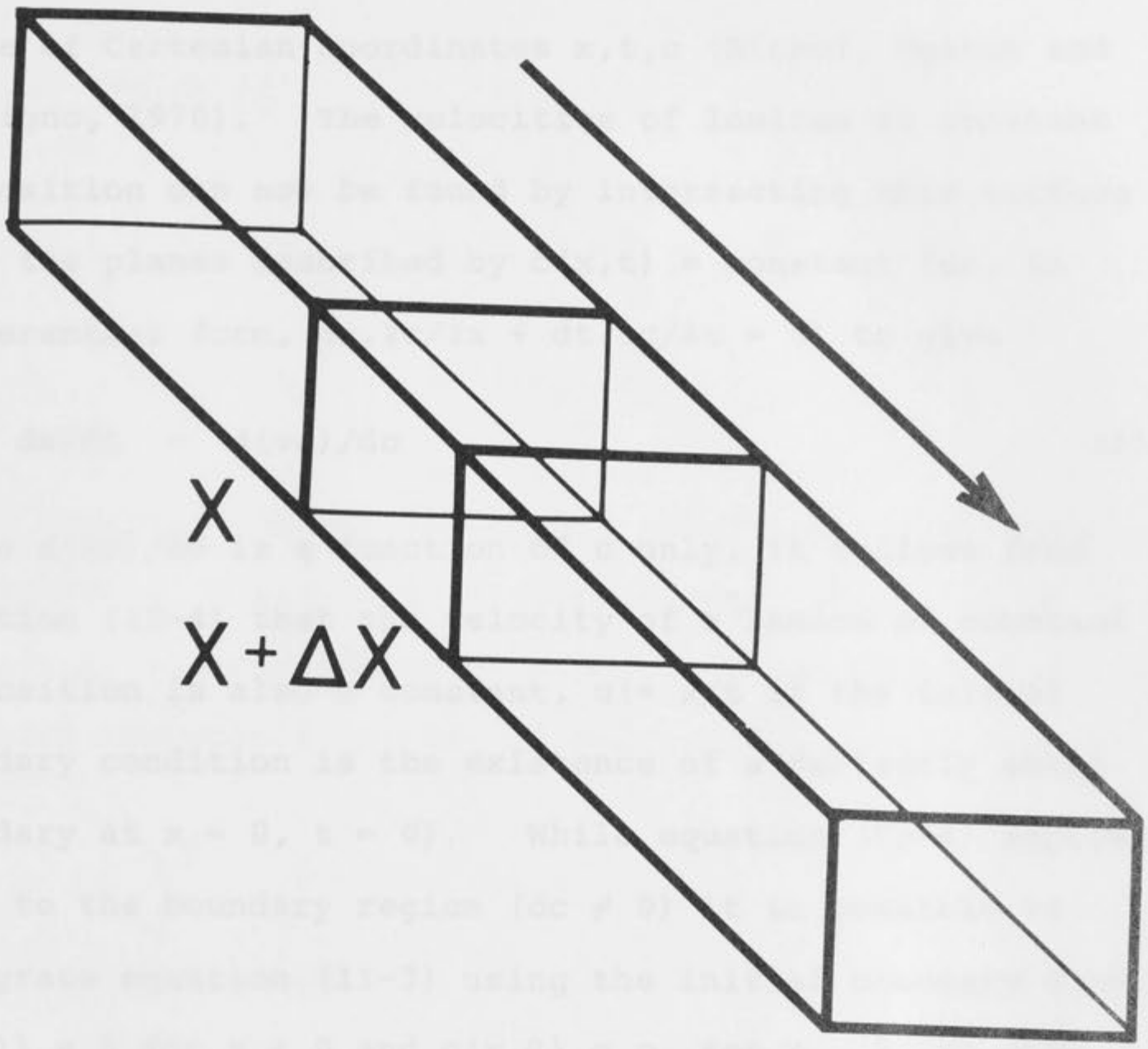


FIGURE II-1

Mass migration in a cell of uniform cross section. A diagram showing the direction of migration (→) and the lamina for which the conservation-of-mass equation is derived.

$$\partial c/\partial t + d(vc)/dc \cdot \partial c/\partial x = 0 \qquad \text{(II-3)}$$

Equation (II-3) describes a surface in the three dimensional space of Cartesian coordinates x,t,c (Nichol, Ogston and Rescigno, 1970). The velocities of laminae of constant composition can now be found by intersecting this surface with the planes described by c(x,t) = constant (or, in differential form, $dx.\partial c/\partial x + dt.\partial c/\partial t = 0$) to give

$$dx/dt = d(vc)/dc \qquad \text{(II-4)}$$

Since d(vc)/dc is a function of c only, it follows from equation (II-4) that the velocity of a lamina of constant composition is also a constant, u(= x/t if the initial boundary condition is the existence of a perfectly sharp boundary at x = 0, t = 0). While equation (II-4) applies only to the boundary region ($dc \neq 0$) it is possible to integrate equation (II-3) using the initial boundary condition $c(x,0) = 0$ for $x \leq 0$ and $c(x,0) = c_0$ for $x > 0$, to give a solution describing both the plateaux and the boundary (Nichol *et al.*, 1970). The solution (in differential form) written in terms of the variable u (Nichol and Ogston, 1965a) is

$$udc - d(vc) = 0 \qquad \text{(II-5)}$$

For a single non-interacting solute whose velocity is independent of concentration, equation (II-5) becomes (u-v)dc = 0 which has two possible solutions. The first, dc = 0, describes the solvent and original solution plateaux, while the second, u = v, refers to the sharp boundary separating them. This simple formulation may now be extended to systems for which the single solute exists as a series of polymeric forms in rapid equilibrium.

(b) Constituent quantities

For a series of successive association equilibria,

$$A_1 + A_1 \rightleftharpoons A_2 \quad ; \quad K_2 = c_{A_2}/c_{A_1}^2$$

$$A_{i-1} + A_1 \rightleftharpoons A_i \quad ; \quad K_i = c_{A_i}/c_{A_{i-1}} c_{A_1} \qquad \text{(II-6)}$$

$$A_{n-1} + A_1 \rightleftharpoons A_n \quad ; \quad K_n = c_{A_n}/c_{A_{n-1}} c_{A_1}$$

there coexists monomer and higher polymers, which constitute, nevertheless, a single Gibbs component. The total or *constituent* concentration at a point, independent of the state in which the solute exists, is defined, on a weight scale as

$$\bar{c} = \sum_{i=1}^{n} c_{A_i} \qquad \text{(II-7)}$$

The corresponding constituent velocity $\bar{v}$, defined as the weight-average of all the velocities v_i,(i=1,2,...,n) characteristic of each species, A_i, is given by

$$\bar{v} = \sum_{i=1}^{n} v_i c_{A_i}/\bar{c} \qquad \text{(II-8)}$$

Substitution of these constituent quantities in equation (II-5) yields the statement of conservation of mass of component A,

$$u d\bar{c} - d(\bar{v}\bar{c}) = 0 \qquad \text{(II-9)}$$

(c) <u>A general description of migration patterns: the determination of the weight-average velocity</u>

Substitution of equations (II-7) and (II-8) into equation (II-9) yields

$$u\sum_{i} dc_{A_i} - d\left(\sum_{i} v_i c_{A_i}\right) = 0 \qquad \text{(II-10)}$$

which, assuming the v_i are independent of total concentration, may be rewritten as

$$\sum_{i} (u-v_i)dc_{A_i} = 0 \qquad \text{(II-11)}$$

From equations (II-6) describing a series of successive association equilibria it may be seen that

$$c_{A_i} = \left\{\prod_{j=1}^{i} K_j\right\} c_{A_1}^{i} \qquad \text{(II-12)}$$

which on substitution into equation (II-11) gives

$$dc_{A_1}\sum_{i} (u-v_i)i\left\{\prod_{j=1}^{i} K_j\right\} c_{A_1}^{i-1} = 0 \qquad \text{(II-13)}$$

This equation has two solutions. The first, $dc_{A_1} = 0$, describes the region of pure solvent (the solvent plateau) where all c_{A_i} equal zero, and the solution plateau in which each c_{A_i} equals $c^0_{A_i}$, the concentration of A_i in the original mixture. The second solution describes the region separating these plateaux,

$$u = \sum_{i} v_i i\{\prod_{j=1}^{i} K_j\} c_{A_1}^{i-1} \Big/ \sum_{i} i\{\prod_{j=1}^{i} K_j\} c_{A_1}^{i-1} \qquad \text{(II-14)}$$

This equation will be used later to discuss the detailed form of the boundary, referred to as a reaction boundary, (Longsworth, 1943), in which all species A_i coexist in rapid equilibrium. For the present, it suffices to note that it may be written as $u = d\bar{v}\bar{c}/d\bar{c}$ and integrated with respect to total concentration from solvent to solution plateau to find the median bisector $\bar{u}$ of the reaction boundary

$$\bar{u} = \int_0^{\bar{c}^0} u d\bar{c} \Big/ \int_0^{\bar{c}^0} d\bar{c} = \int_0^{\bar{c}^0} d(\bar{v}\bar{c}) \Big/ \int_0^{\bar{c}^0} d\bar{c} = \bar{v} \qquad \text{(II-15)}$$

Equation (II-15) shows that the weight-average velocity $\bar{v}$, corresponding to the initial concentration $\bar{c}^0$, is given by the value of u which defines the median bisector (called the centroid by Longsworth, 1943) of the reaction boundary in a time-normalised migration pattern. In practice, plots of $\bar{c}$ *vs.* x are recorded at different times, and the weight-average velocity is given by the rate of movement of the median bisector of the reaction boundary.

(d) A more detailed description of the reaction boundary

While equations (II-7), (II-12) and (II-14) may be used to construct numerical examples of reaction boundaries for any selected values of v_i, K_j and n, a considerable simplification is achieved by restricting attention to systems in which the amounts of intermediate polymers are negligible. These systems (using the notation of Chapter I)

may be described by $nA \rightleftharpoons C$, and with $A_1 = A$ and $X' = c_C/c_A^n = \prod_{j=1}^{n} K_j$, equation (II-14) reduces to

$$u = (v_A + v_C n c_A^{n-1} X')/(1 + n c_A^{n-1} X') \qquad \text{(II-16)}$$

This equation is directly applicable to all cases in which the highest polymer formed is a dimer, n = 2, (for example, haemoglobin, which is studied in more detail in Chapter IV) and it may also apply, when n > 2, to systems such as β-lactoglobulin A near pH 4.6, for which the relative amounts of polymers of size intermediate between dimer (36,000) and octamer (144,000) appear to be small (Townend and Timasheff, 1960; McKenzie, Sawyer and Smith, 1967). In the present work, therefore, it is appropriate to examine equation (II-16) in detail. Rearranging it to yield an expression for c_A, and hence $c_C (= X' c_A^n)$, and adding the results gives

$$\bar{c} = (1/X')^{1/(n-1)} \{ [(v_A - u)/n(u - v_C)]^{1/(n-1)} + [(v_A - u)/n(u - v_C)]^{n/(n-1)} \} \qquad \text{(II-17)}$$

which may be differentiated with respect to u to yield

$$d\bar{c}/du = (v_C - v_A) \{ [(v_A - u)/n(u - v_C)]^{(2-n)/(n-1)} + n[(v_A - u)/n(u - v_C)]^{1/(n-1)} \}/n(n-1)(u - v_C)^2 (X')^{1/(n-1)} \qquad \text{(II-18)}$$

Equation (II-18) may be used to construct theoretical time-normalised schlieren patterns of $d\bar{c}/du$ *vs.* u.

(Figure (II-2) provides numerical examples of such plots. It is noted that in contrast to the behaviour predicted for a dimerising system, the curve for n = 4 exhibits a minimum. Gilbert (1955, 1959) obtained an expression for $d^2\bar{c}/du^2$ which, when equated to zero, showed that a minimum occurred in the $d\bar{c}/du$ *vs.* u plot at a value of u_{min} given by

$$(u_{min}-v_A)/(v_C-v_A) = (n-2)/3(n-1) \quad ; \quad n > 2 \qquad \text{(II-19)}$$

He concluded from equation (II-19) that the schlieren pattern for a dimerising system (n = 2) would exhibit no minimum, but when n was greater than 2 the pattern would appear bimodal. The area enclosed by such a pattern and the base-line between $u = v_A$ and $u = u_{min}$ gives $\bar{c}_{min}$ at u_{min}. Equations (II-17) and (II-19) written for the point (u_{min}, c_{min}) may then be combined to yield, on rearrangement,

$$X' = \bar{c}_{min}^{1-n}(n-2)\{2(n^2-1)\}^{n-1}/\{n(2n-1)\}^n \qquad \text{(II-20)}$$

This equation states clearly the conclusion, which was originally drawn by Gilbert (1959), that the area under the back portion of the bimodal reaction boundary belonging to a system of the type $nA \rightleftharpoons C$ (n > 2), will remain constant as $\bar{c}^0$ is varied provided $\bar{c}^0 > \bar{c}_{min}$. This is illustrated by the broken vertical lines in Figure (II-2). Moreover, if n is known, equation (II-20) may be used to calculate the equilibrium constant X' for these systems from an experimentally observed $\bar{c}_{min}$.

The above description of spread reaction boundaries (illustrated in Figure II-2) applies to the sedimentation

FIGURE II-2

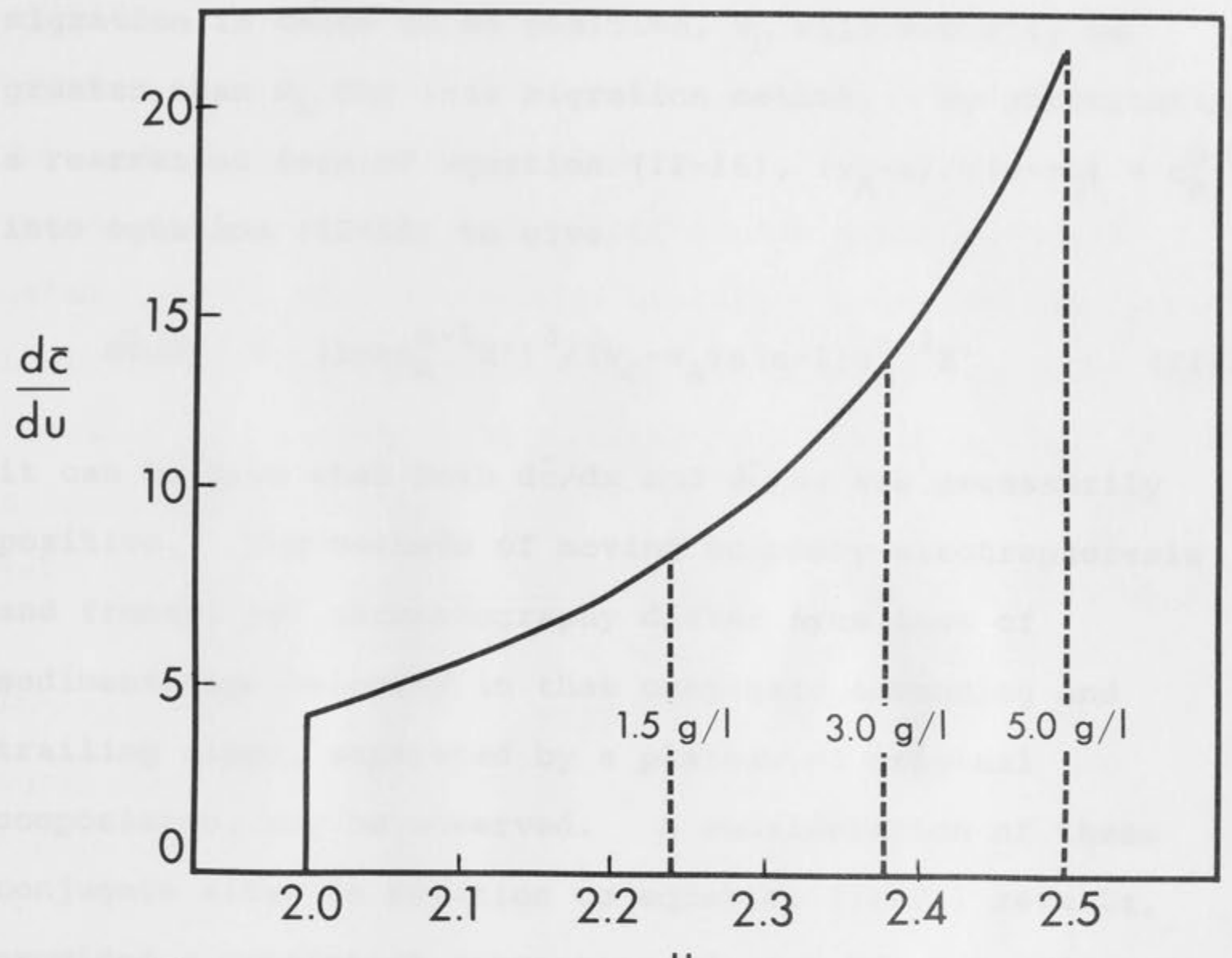
Mass migration of a solute which is rapidly self-interacting according to the scheme $nA \rightleftharpoons C$.

Theoretical time-normalised schlieren patterns of $d\bar{c}/du$ *vs.* u, without diffusion.

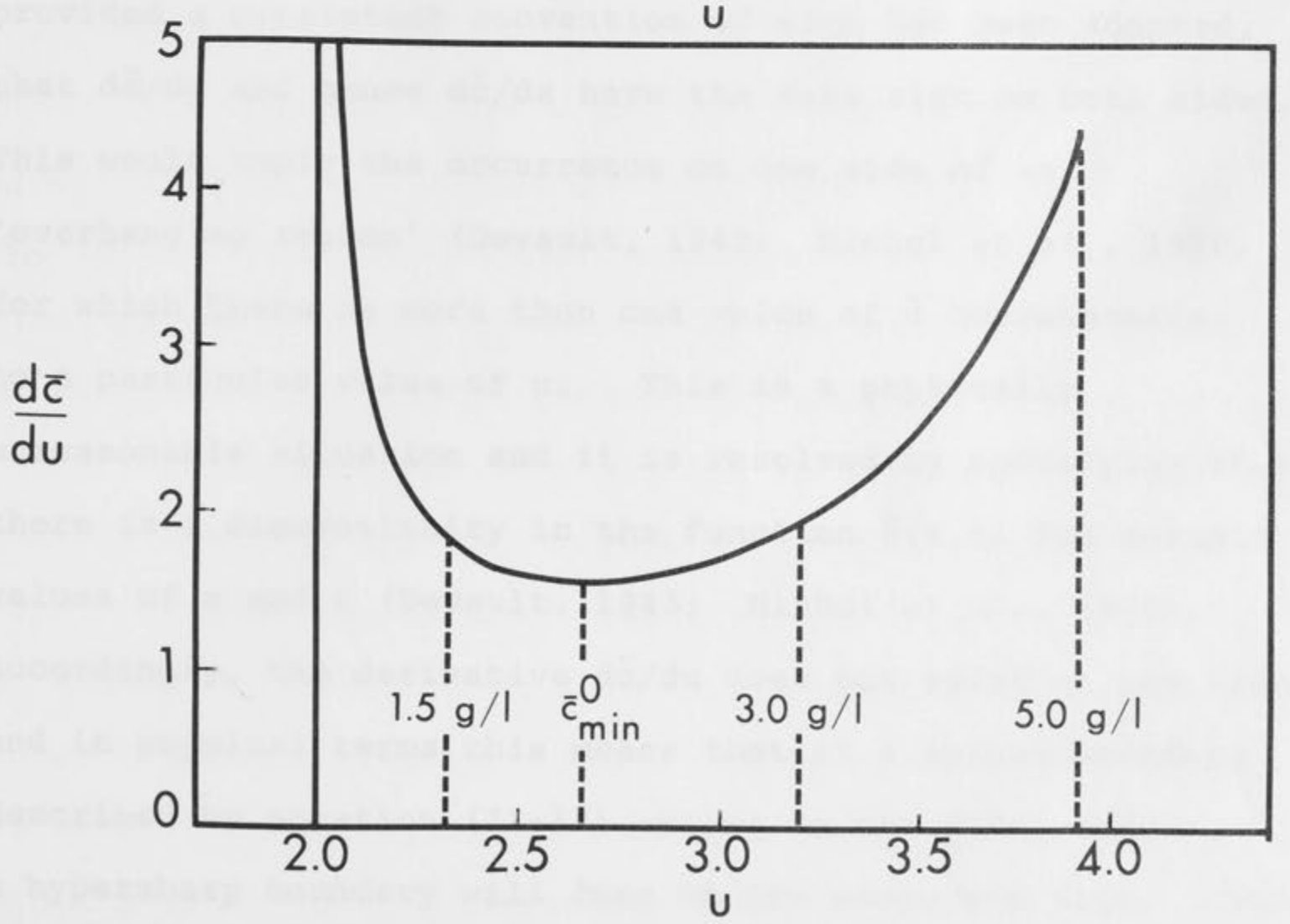
(a) A monomer-dimer system (n = 2), with $v_A = 2$, $v_C = 2 \times 2^{2/3}$, and $X' = 0.1\ l.g^{-1}$

(b) A monomer-tetramer system (n = 4), with $v_A = 2$, $v_C = 2 \times 4^{2/3}$, and $X' = 0.01\ l^3.g^{-3}$.

Each vertical dotted line indicates the value of u at which the boundary terminates for a solution of a given initial total concentration $\bar{c}_o$.


20
15
10
5
0
dc̄/du
1.5 g/l
3.0 g/l
5.0 g/l
2.0
2.1
2.2
2.3
2.4
2.5
u

(a)


5
4
3
2
1
0
dc̄/du
1.5 g/l
c̄0min
3.0 g/l
5.0 g/l
2.0
2.5
3.0
3.5
4.0
u

(b)

velocity of a polymerising system. If the direction of migration is taken to be positive, v_C will normally be greater than v_A for this migration method. By substituting a rearranged form of equation (II-16), $(v_A-u)/n(u-v_C) = c_A^{n-1}X'$, into equation (II-18) to give

$$d\bar{c}/du = (1+nc_A^{n-1}X')^3/(v_C-v_A)n(n-1)c_A^{n-2}X' \qquad \text{(II-21)}$$

it can be seen that both $d\bar{c}/dx$ and $d\bar{c}/du$ are necessarily positive. The methods of moving boundary electrophoresis and frontal gel chromatography differ from that of sedimentation velocity in that conjugate advancing and trailing sides, separated by a plateau of original composition, may be observed. A consideration of these conjugate sides in relation to equation (II-21) reveals, provided a consistent convention of sign has been adopted, that $d\bar{c}/du$ and hence $d\bar{c}/dx$ have the same sign on both sides. This would imply the occurrence on one side of an 'overhanging region' (Devault, 1943; Nichol *et al.*, 1970) for which there is more than one value of $\bar{c}$ corresponding to a particular value of u. This is a physically unreasonable situation and it is resolved by specifying that there is a discontinuity in the function $\bar{c}(x,t)$ for certain values of x and t (Devault, 1943; Nichol *et al.*, 1970). Accordingly, the derivative $d\bar{c}/du$ does not exist on one side, and in physical terms this means that if a spread boundary described by equation (II-17) arises on one side, then a hypersharp boundary will form on the conjugate side. This type of non-enantiography has been observed in the moving boundary electrophoresis of β-lactoglobulin (Tombs, 1957) and

it provides an excellent qualitative indication of the existence of an interaction. Moreover, a hypersharp boundary must move with the weight-average velocity corresponding to the initial concentration $\bar{c}^0$ and is potentially capable of providing a more accurate means of determining $\bar{v}$ than the median bisector of the spread reaction boundary on the conjugate side. In practice, however, the tendency of a boundary to hypersharpen is opposed by diffusional spreading.

(e) The use of weight-average velocities in determining X'

For the system $nA \rightleftharpoons C$, a direct rearrangement of equation (II-8), together with the definition of the equilibrium constant $X' = c_C/c_A^n$ yields

$$X' = \bar{c}^{1-n}(v_A-v_C)^{n-1}(v_A-\bar{v})/(\bar{v}-v_C)^n \qquad \text{(II-22)}$$

where $\bar{v}$, determined from either conjugate side, refers to the concentration $\bar{c}$ in the plateau separating them. Equation (II-22) may be used to estimate X' (even when n = 2) provided estimates of v_A and v_C may be obtained. In general, an estimate of v_A may be obtained by extrapolating values of $\bar{v}$ to infinite dilution (n > 1), and in sedimentation velocity the value of v_C (i.e. s_C) may be determined in one of two ways. First, geometrical reasoning may be used to relate s_C and s_A, for example $s_C = n^{2/3}s_A$. Secondly, for cases in which a bimodal reaction boundary is observed (n > 2) the value of u_{min} may be substituted into equation (II-19) to yield a value of s_C (Nichol and Bethune, 1963). In this connection it must be stressed that the rates of movement of the two peaks in a bimodal boundary do not correspond

to s_A and s_C (Gilbert and Gilbert, 1962; Gilbert, 1959).

The other type of migration experiment employed in this study is frontal analysis chromatography using Sephadex gels as the stationary phase. The information available to this experimenter was an elution profile of $\bar{c}$ *vs.* the elution volume V (*cf.* Brumbaugh and Ackers, 1967). It has been shown that the basic equations derived in this section are still applicable to an interpretation of elution profiles, provided velocity terms are replaced directly by elution volumes (Gilbert, 1966a; Nichol, Ogston and Winzor, 1967). An example of the validity of this transformation is given in the next Chapter. Accordingly, the weight-average elution volume $\bar{V}$ appropriate to equation (II-22) may be determined from the elution profile using the equation

$$\bar{V} = \int_0^{c_0} V d\bar{c} \Big/ \int_0^{c_0} d\bar{c} \simeq \sum V \Delta\bar{c}/\bar{c}^0 \qquad \text{(II-23)}$$

The problem of determining V_A and V_C remains. The method of gel chromatography offers the advantage that a gel type may be chosen to exclude the polymer C, permitting V_C to be identified with the void volume of the column. The elution volume of the monomer, V_A, may be obtained either by extrapolation to infinite dilution, or from a single experiment using the procedure outlined by Winzor, Loke and Nichol (1967). These authors define $\bar{V}$ in accordance with equation (II-8) and a z-average elution volume V_z as follows,

$$\bar{V} = \{c_A^0 V_A + X'(c_A^0)^n V_C\}/\{c_A^0 + X'(c_0^A)^n\} \qquad \text{(II-24a)}$$

$$V_z = \{c_A^0 V_A^2 + X'(c_A^0)^n V_C^2\} / \{c_A^0 V_A + X'(c_0^A)^n V_C\} \qquad \text{(II-24b)}$$

From equation (II-24a) it follows that $X'(c_A^0)^{n-1} = (V_A - \bar{V})/(\bar{V} - V_C)$ which, on substitution into (II-24b) yields

$$V_z = V_A + V_C - (V_A V_C / \bar{V}) \qquad \text{(II-25)}$$

Therefore, if V_z can be evaluated from the elution profile, V_A follows directly from equation (II-25). Winzor *et al.* (1967) have shown that V_z closely approximates the experimentally available quantity V_z^* defined by

$$V_z^* = \sum V^2 \Delta\bar{c} / \sum V \Delta\bar{c} \qquad \text{(II-26)}$$

(f) Summary

This section has shown that two expressions, equation (II-20) and (II-22), are valuable in determining the self-interaction constant X'. It is pertinent, therefore, to comment on the assumptions used in the derivation of these equations in relation to the migration methods which are actually used in this study, viz., sedimentation velocity and frontal chromatography. Although the rectangular approximation is only imperfectly fulfilled in sedimentation velocity, Goldberg (1953) has shown that $\bar{s}$ (equation II-22) may be reliably obtained from the rate of movement of the square root of the second moment of the entire reaction boundary (Nichol, Bethune, Kegeles and Hess, 1964). The effect of radial dilution on the use of equation (II-20) to estimate X' has been considered by Fujita (1962) and Nichol *et al.* (1964), who showed that

$$X' = \bar{c}_{min}^{1-n}(n-2)\psi\{n(2n-1) + (n-2)\psi\}^{n-1}/\{n(2n-1)\}^{n} \quad \text{(II-27a)}$$

$$\text{where } \psi = \{1-s_C(n-2)\tau/3ns_A\}/\{1+\tau(2n-1)/3\} \quad \text{(II-27b)}$$

$$\text{and } \tau = 2s_A\omega^2 t \quad \text{(II-27c)}$$

The limit of ψ as t approaches zero is unity, and equation (II-27a) reduces to equation (II-20). It has been shown that use of the latter equation leads to a trivial error in X' (Nichol and Winzor, 1972). The rectangular approximation is, of course, valid in the case of frontal gel chromatography conducted on a column of uniform cross-sectional area.

In the derivation of the continuity equations (II-5) and (II-9), diffusional flows were neglected. A combination of Fick's first and second laws of diffusion, together with an assumed absence of coupled flows, shows that the terms which account for diffusion are $D_A(\partial^2 c_A/\partial x^2)_t$ and $D_C(\partial^2 c_C/\partial x^2)_t$. Inclusion of these second order differential terms in the continuity equation makes it extremely difficult to solve analytically, although numerical solutions are still possible (Cox, 1967, 1969). Figure (II-3), taken from Cox (1969) illustrates the effect of diffusional spreading in sedimentation velocity patterns for a monomer-hexamer system. The pattern resembles an experimental result more closely than the asymptotic patterns shown in Figure (II-2), but it is clear that the basic predictions of equation (II-20) are unaltered. Firstly, the area of the back portion of the bimodal reaction boundary remains constant with increasing total concentration, and secondly the value of x_{min}

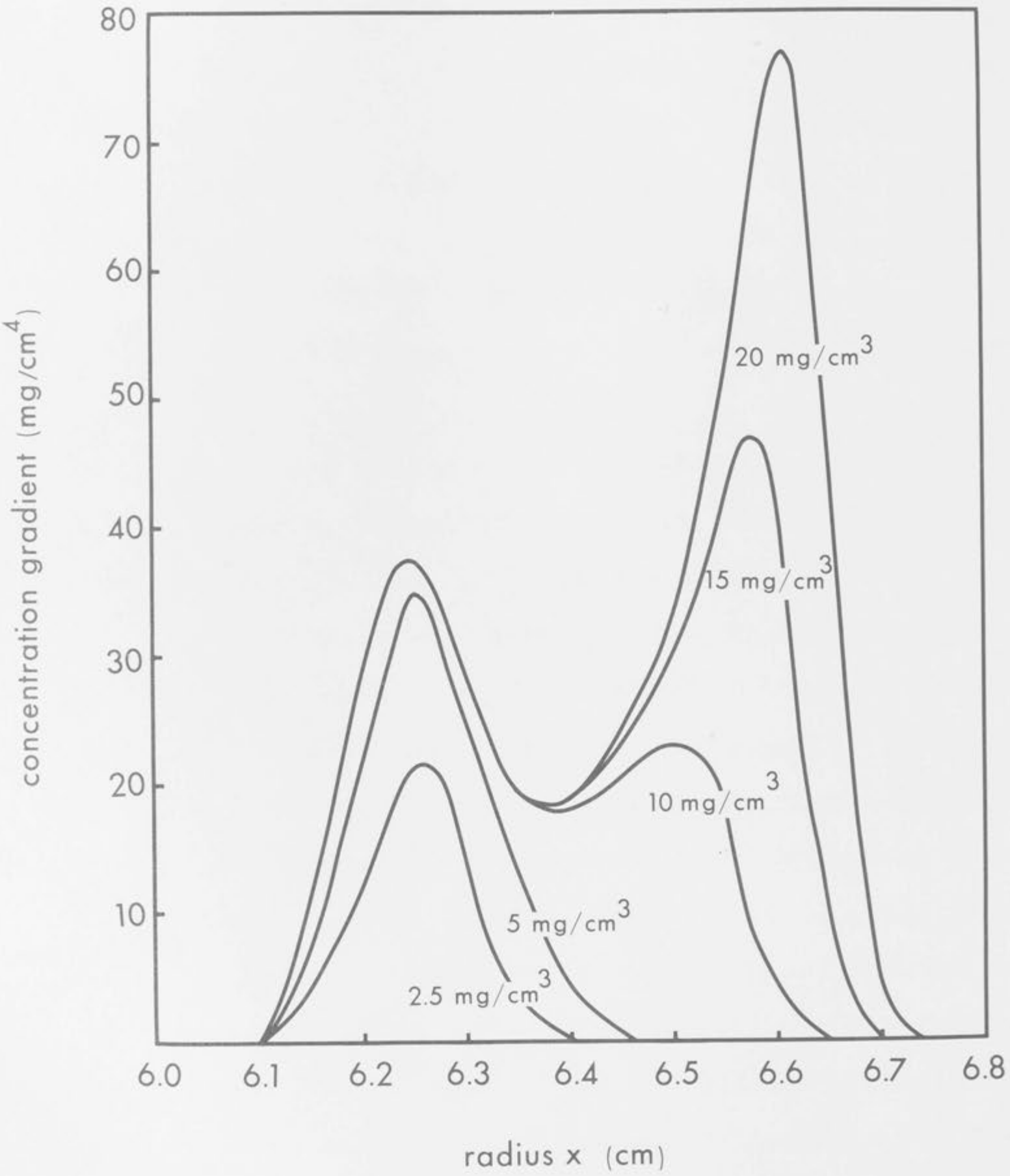

80
70
60
50
40
30
20
10
concentration gradient (mg/cm^4)
20 mg/cm^3
15 mg/cm^3
10 mg/cm^3
5 mg/cm^3
2.5 mg/cm^3
6.0
6.1
6.2
6.3
6.4
6.5
6.6
6.7
6.8
radius x (cm)

FIGURE II-3

A theoretical sedimentation velocity pattern, incorporating diffusional spreading, of a monomer-hexamer system (n = 6). Values of parameters needed for the simulation were:

rotor speed, 59,780 r.p.m.

meniscus position 6.085 cm

$s_A = 6.0$ S

$s_C = n^{2/3} s_A$

$D_A = 8.0 \times 10^{-7}$ cm^2 sec^{-1}

$D_C = D_A n^{-1/3}$

$X' = 1.6 \times 10^{-5}$ l^5.g^{-5}

$t = 1200$ sec

The initial solute concentrations, $\bar{c}_o$, ranging from 2.5 to 20 mg/ml, are indicated in the diagram.

(Adapted from Cox, 1969).

calculated on the basis of a theory neglecting diffusional flows continues to locate the position of the minimum. (This value of x_{min} was found by calculating $u_{min}(s_{min})$ from equation (II-19) and hence x_{min} from $s_{min} = \ln(x_{min}/x_{meniscus})/\omega^2 t$). Thus it appears that the use of equation (II-20) to evaluate X' will be little affected by the occurrence of diffusional spreading. In relation to the applicability of equation (II-22) it need only be pointed out that the weight-average position of a boundary is unaffected by diffusional spreading. Zimmerman, Cox and Ackers (1971) have considered the problem of axial dispersion in gel chromatography and concluded that this analogue to diffusional spreading may alter the area distribution under the bimodal reaction boundary from that predicted by equation (II-20). These workers also questioned the use of the centroid (median bisector) of the spread reaction boundary in determining X'. However, inspection of Figure 9 of Zimmerman and Ackers (1971) reveals that while the value of the total concentration corresponding to the centroid may be affected by axial dispersion, the corresponding abscissa value (the weight-average elution volume) is not. Thus, the reservations of Zimmerman *et al.* (1971) apply to the use of the centroid *concentration* in determining X', which is indeed a hazardous procedure. They do not apply to the use of the weight-average elution volume in equation (II-22).

In writing equation (II-11) it was assumed that the velocities of the individual species were constants independent of total concentration, but in practice the

sedimentation coefficients and elution volumes of globular proteins exhibit a linear concentration dependence (e.g. Svedberg and Pedersen, 1940; Winzor and Nichol, 1965; Chiancone, Gilbert, Gilbert and Kellett, 1968). In sedimentation velocity the effect has been attributed to viscosity and density effects together with a contribution from the backward flow of solvent, (Schachman, 1959); while in gel chromatography it is likely to be osmotic in origin (Edmond, Farquar, Dunstone and Ogston, 1968; Nichol, Sawyer and Winzor, 1969; Ogston, 1970; Nichol, Janado and Winzor, 1973). The consequences of a linear concentration dependence in the migration of a non-self-interacting solute are well understood: it results in a hypersharpening on one side and spreading on the conjugate side without introducing assymetry into the spread boundary (Fujita, 1956; Winzor and Nichol, 1965). Less is known about its effects when the solute is chemically interacting, although Gilbert (1963) has derived the analogue to equation (II-14) on the basis that $v_i = v_i^0(1-k\bar{c})$. In the analysis of a mixture of two components which do not interact chemically, the operation of a Johnston-Ogston effect (1946) arising as a result of concentration-dependent migration should be taken into account. In the present context, it is uncertain whether this effect will alter the value of $\bar{c}_{min}$ appropriate to equation (II-20). It is unlikely, however, that a serious error in the estimate of X' would arise as a consequence of neglecting this effect in the study of globular proteins. Although concentration-dependence of the v_i may affect the form of a reaction boundary, $\bar{v}$ may be reliably estimated again from the median bisector (or square

root of the second moment) of the boundary, and hence equation (II-22) is still directly applicable. Values of v_A and v_C referring to the initial concentration $\bar{c}^0$ may be estimated from

$$v_i = v_i^0(1-k\bar{c}^0) \qquad \text{(II-28)}$$

where the coefficient k is assumed identical for each species i (Gilbert, 1963; Chiancone *et al.*, 1968; Ackers, 1967) and is positive for sedimentation velocity and negative for frontal gel chromatography.

Finally, it is noted that the differentiation leading to equation (II-13) implies that all the K (and hence X' for $nA \rightleftharpoons C$) are constant, which further implies, for polymerisations with finite enthalpy and/or volume changes that the temperature and pressure are constant during mass migration (Chapter I). This offers no problem in gel chromatography, but in sedimentation velocity experiments the centrifugal field creates a pressure gradient $dP = x\omega^2\rho dx$, where ω is the angular velocity and ρ the density of the solution. Since $(\partial \ln X/\partial P)_T = -\Delta V/RT$ it follows that

$$X'(x_1) = X'(x_2)e^{-\{\Delta V\omega^2\rho(x_1^2-x_2^2)\}/2RT} \qquad \text{(II-29)}$$

and that X' is therefore a function of radial distance. In their studies of the sedimentation of the polymerising myosin system, Josephs and Harrington (1968) have shown that the nature of the migration pattern may be seriously affected by this pressure dependence. It would seem unwise,

therefore, to use sedimentation velocity alone to characterise an interaction whose molar volume change is large. In this situation, a comparison with results obtained by other mass migration methods (or equilibrium methods) is desirable.

2. Studies with β-lactoglobulin A in aqueous and deuterium oxide media.

(a) The statement of the problem

Several workers have investigated the polymerisation behaviour of different genetic variants of β-lactoglobulin in aqueous solution. Above pH 5.2 all the variants exist predominantly as dimers whose tendency to dissociate into monomeric units (18,000 daltons) becomes significant only as the pH is decreased below a value of approximately 3.5 (Townend and Timasheff, 1960; Townend, Weinberger and Timasheff, 1960). In contrast to the other known variants, β-lactoglobulin A dimers associate pronouncedly to form octamer units at an intermediate pH of *ca.* 4.6. Equilibrium studies employing light-scattering (Kumosinski and Timasheff, 1966) and sedimentation equilibrium (Adams and Lewis, 1968) have indicated that a series of polymers may coexist in the equilibrium mixture at pH 4.6. However, other studies (Townend, Winterbottom and Timasheff, 1960; McKenzie, Sawyer and Smith, 1967; Gilbert, 1963) strongly suggest that the dimeric and octameric forms predominate, a conclusion supported by the low angle X-ray studies of Witz, Timasheff and Luzzati (1964). The failure of other variants to form octamers to any appreciable extent at pH 4.6 has provided indirect evidence of the groups involved in the formation of the β-lactoglobulin A octamer. Monomer

units of the A variant differ from those of the B variant by the possession of an extra aspartic acid residue, while the monomers of the "Droughtmaster" variant differ from those of the A variant by the occurrence of sialic acid and hexosamine bound to a small peptide containing arginine, serine and glutamic acid residues among others (Bell, McKenzie and Shaw, 1966; Bell, McKenzie and Murphy, 1966). These findings have led to the speculation that hydrogen bonding between aspartic and glutamic acid residues may be important in the dimer-octamer association (Armstrong and McKenzie, 1967). A measure of support for this suggestion has come from the finding by Armstrong and McKenzie (1967) that modification of the carboxyl groups of β-lactoglobulin A with a water-soluble carbodiimide almost completely prevents the association reaction, without apparently affecting the tertiary structure of the protein. This evidence is not conclusive, however, because the net electrostatic charge borne by the protein may be markedly altered by the modification. The dimer-octamer association of β-lactoglobulin A provides an excellent example of a self-interaction whose equilibrium constant X' is affected markedly by a variation in temperature; the associated enthalpy change being approximately -40 to -60 Kcals/mole (McKenzie *et al.*, 1967). This finding that association is favoured by lowering the temperature indicates that hydrophobic interactions (Kauzmann, 1959) do not contribute significantly to octamer formation. While little information is available on the effects of changing variables other than temperature, for example, pH, ionic strength and solvent

environment, on the extent of this association, such studies may be performed quite readily, using, for example, equations (II-20) and (II-22) in the analysis, since the system is essentially of the type $nA \rightleftharpoons C$. Moreover, they may provide information which is of considerable value in a general context. In particular it was felt that it would be of interest to examine the effect of deuterium oxide on X' for reasons which will now be discussed.

The signs and approximate magnitudes of the enthalpy changes accompanying the self-associations of several proteins have been determined by studying the variation of X' with temperature. These enthalpy changes have been shown to be near zero or positive for haemoglobin (Rossi-Fanelli, Antonini and Caputo, 1964), glutamate dehydrogenase (Reisler and Eisenberg, 1971) and insulin (Jeffrey and Coates, 1966) and since this implies that the associations are largely entropically driven, it has been suggested that hydrophobic bonding may play an important role in stabilising the polymers. From studies on the effect of deuterium oxide on the critical micelle concentration of ionic detergents, and measurements of the free energies of transfer of model compounds (including some amino acids) from H_2O to D_2O, Kresheck, Schneider and Scheraga (1965) predicted that hydrophobic bonds between protiated amino acid side chains would be stronger in D_2O than in H_2O. Subsequently D_2O was found to enhance the association of mitotic spindle fibres (Inoué, Sato, Kane and Stephens, 1965), tobacco mosaic virus protein (Paglini and Lauffer, 1968), glutamate dehydrogenase (Henderson, Henderson and Woodfin, 1970), chymotrypsin (Aune, Goldsmith

and Timasheff, 1971) and algal phycocyanin (Lee and Berns, 1968). In discussing their results with phycocyanin these last authors claim that "the use of deuterium oxide is a convenient method for testing the relative importance of hydrophobic forces in stabilising various protein aggregates". This view was expanded in a later monograph (Berns, 1971). If this hypothesis were correct, it might be expected that the extent of the dimer-octamer association of β-lactoglobulin A would not be significantly increased in deuterium oxide and it was for this reason that studies on β-lactoglobulin A in deuterium oxide were undertaken.

(b) Results

The details of the experimental methods used in this investigation may be found in Chapter V, and only those aspects which are required for an understanding of the results will be presented in this Chapter. All studies were performed with recrystallised β-lactoglobulin A dissolved in either aqueous acetate buffer (0.1 M sodium acetate, 0.088 M acetic acid, pH 4.65, I = 0.1) or a buffer in which glass-distilled water was replaced by heavy water. Measurement of pH was made at 20° with a Radiometer pH meter equipped with standard glass and calomel electrodes. With D_2O solutions, the meter reading was adjusted to a value of 4.25 by the addition of acetic acid, since the pD of D_2O solutions equals the meter reading plus 0.4 (Glascoe and Long, 1960). The procedure accounts for the difference in the ionisation constants of H_2O and D_2O, and yields a pD of 4.65.

(i) Sedimentation velocity studies. Sedimentation velocity patterns obtained at 12° and 59,780 r.p.m. with solutions of β-lactoglobulin A in aqueous and D_2O media are compared in Figure (II-4). In accordance with equation II-19, with n = 4, bimodal reaction boundaries were observed in each medium. However, the relative proportion of the total area associated with the trailing portion of the boundaries is greater in the aqueous environment (upper pattern), which implies (equation (II-20)) that octamer formation is favoured in deuterium oxide. Similar results were obtained at other temperatures employing a fixed concentration $\bar{c}^0$ = 13 g/l, and the patterns were analysed according to equation (II-20) to estimate the association constant $X'(l^3.g^{-3})$ and hence $\log_{10} X$, where X is expressed in l^3. $moles^{-3}$ to permit later comparison with results reviewed by McKenzie *et al.* (1967). In these terms equation (II-20), with n = 4, becomes $X' = 0.088 - 3\,\bar{c}_{min}$ $(= 4XM_A^{1-n})$, and thus $\log X = 12.01 - 3 \log \bar{c}_{min}$. The results of these calculations are shown in Table (II-1a). Although the unimodal boundary observed in aqueous acetate buffer at 20° could not be resolved, it is clear from the results that X is greater in the heavy water environment at all temperatures studied, and that in both environments it decreases with increasing temperature.

These conclusions are supported by an analysis of the same sedimentation velocity results in terms of weight-average sedimentation coefficients using equation (II-22). The values of $\bar{s}_{20,w}$ reported in Table (II-1b) were obtained by correcting the experimentally observed weight-average

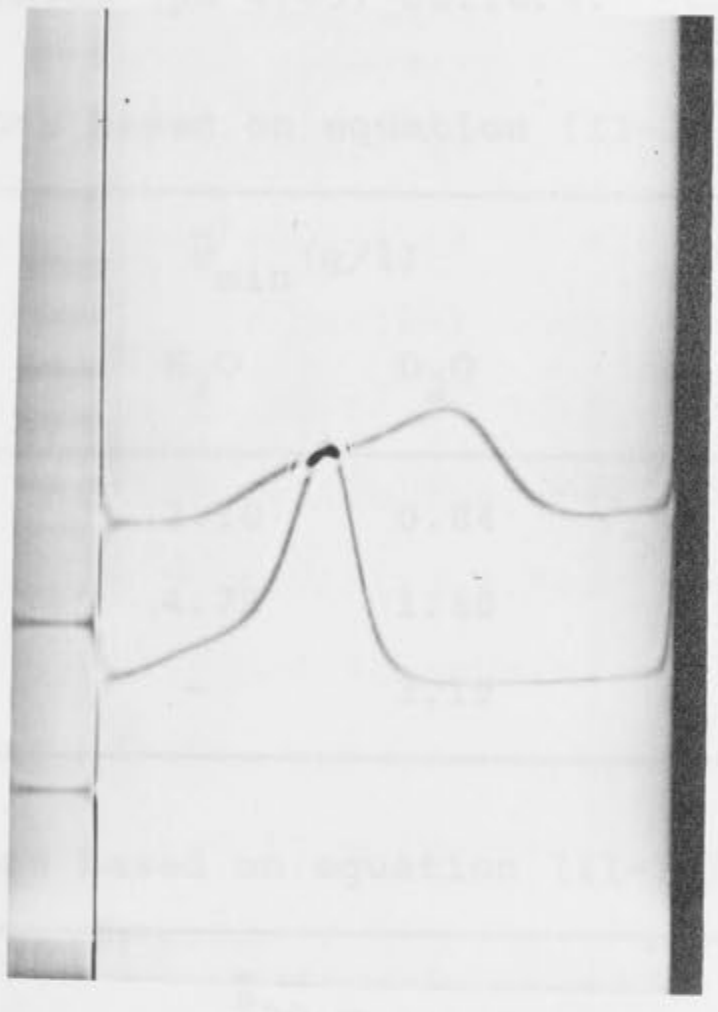


FIGURE II-4

Sedimentation velocity patterns obtained with solutions of β-lactoglobulin A (13 g/l) at 12°. Sedimentation is from left to right. The upper pattern refers to an aqueous acetate buffer (pH 4.65) and the lower pattern to a D_2O medium (pD 4.65).

TABLE II-1

Sedimentation velocity results obtained with bovine β-lactoglobulin A in 0.1 M sodium acetate, aqueous (pH 4.65) and deuterium oxide (pD 4.65) buffers.

(a) Calculations based on equation (II-20)

Temperature	$\bar{c}_{min}$ (g/l)		log X	
(°C)	H_2O	D_2O	H_2O	D_2O
4	2.10	0.84	11.1	12.2
12	4.78	1.80	10.0	11.3
20	-	3.19	-	10.5

(b) Calculations based on equation (II-22)

Temperature	$\bar{s}_{20,w}$		log X	
(°C)	H_2O	D_2O	H_2O	D_2O
4	4.39	4.96	10.3	10.9
12	3.65	4.18	9.6	10.0
20	2.95	3.57	8.7	9.5

values, $\bar{s}_{T,b}$ to water at 20° according to Svedberg and Pedersen (1940),

$$\bar{s}_{20,w} = \bar{s}_{T,b}(\eta_{T,b}/\eta_{20,w})(1-\bar{v}\rho)_{20,w}/(1-\bar{v}\rho)_{T,b} \qquad \text{(II-30)}$$

where $\eta_{T,b}$ refers to the viscosity of buffer b (either aqueous or D_2O) at temperature T; ρ the density of buffer and $\bar{v}$ the partial specific volume of the protein in the appropriate buffer. The partial specific volume of β-lactoglobulin A was taken to be 0.746 ml/g in water (McKenzie *et al.*, 1967), while that for the deuterated protein was obtained by dividing 0.746 by the correction factor 1.015 (Edelstein and Schachman, 1967). The values of $\bar{s}_{20,w}$ obtained in this manner are hypothetical, since the enthalpy change involved in polymerisation is finite, but the correction was necessary because values of s_A^0 and s_C^0 were available only at 20° in water (Gilbert, 1963). It is apparent from equation (II-22) that values of X' are unaffected by such corrections. Accordingly, log X' was calculated by substituting these corrected values into the specific form of equation (II-22):

$$X' = (\bar{c}^0)^{-3}\{(s_A)_{20,w} - (s_C)_{20,w}\}^3$$

$$\{(s_A)_{20,w} - \bar{s}_{20,w}\}/\{\bar{s}_{20,w} - (s_C)_{20,w}\}^4 \qquad \text{(II-31)}$$

In detail the values of $(s_A)_{20,w}$ and $(s_C)_{20,w}$ appropriate to $\bar{c}^0$ were calculated using $(s_A)_{20,w} = 2.87(1 - 0.0058\ \bar{c}^0)$ and $(s_C)_{20,w} = 7.37(1 - 0.0058\ \bar{c}^0)$, respectively. Although the values of log X found by this method (Table II-1b) are consistently lower than those found using equation (II-20),

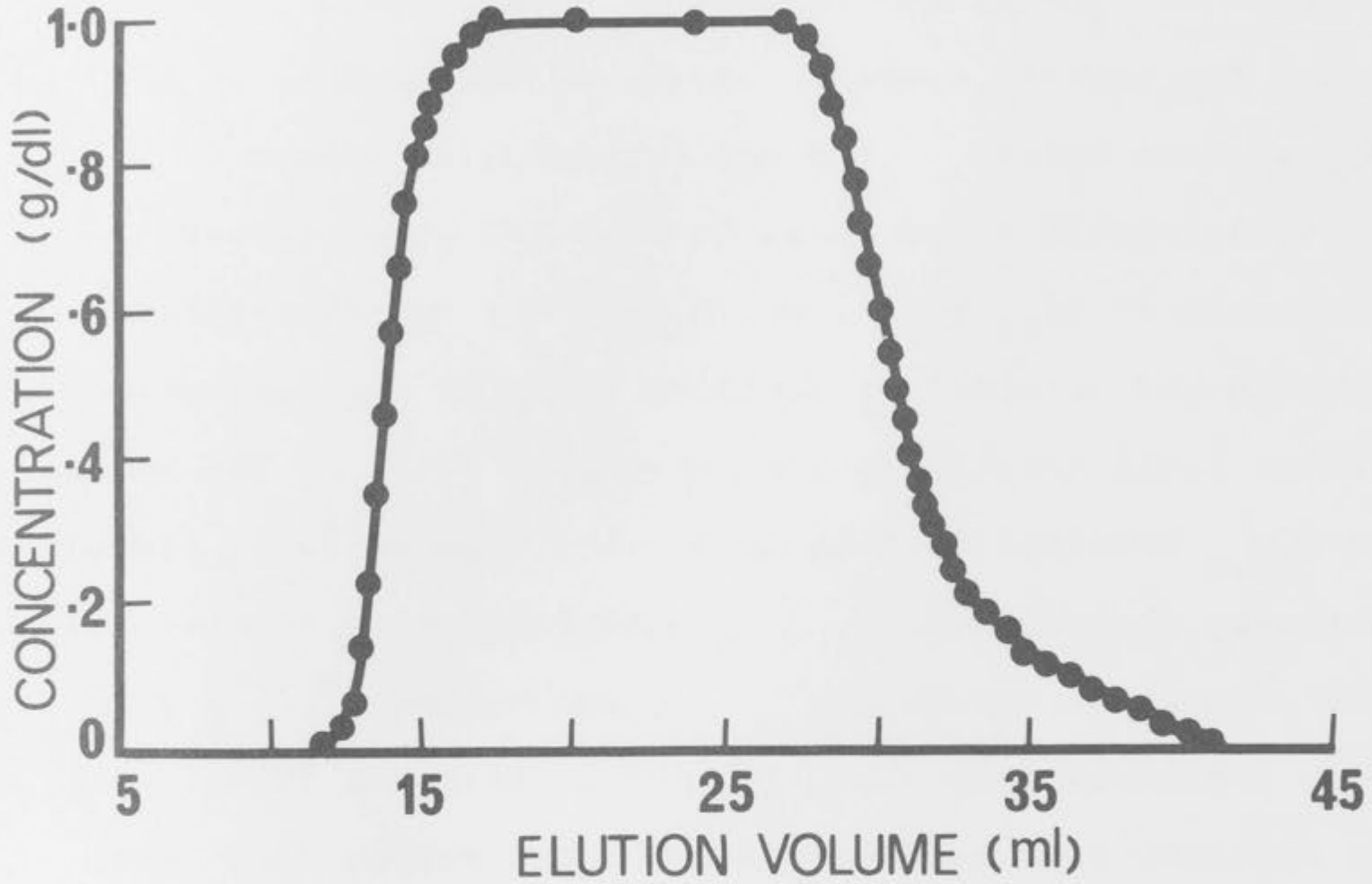


FIGURE II-5

The elution profile obtained from a frontal analysis chromatography experiment conducted with β-lactoglobulin A on Sephadex G-75 (38 cm x 1 cm) at 12°. A D_2O medium (pD 4.65) was used to equilibrate the column and to dissolve the applied protein (17 ml of 10 g/l).

both sets of results support the contention that the extent of asssociation is greater in the deuterium oxide medium.

It was noted earlier that it is unwise to use sedimentation velocity results alone to estimate equilibrium constants, since such estimates may be in error if a significant volume change accompanies the polymerisation (equation (II-29)). The sedimentation equilibrium results of Adams and Lewis (1968) show that values of the apparent weight-average molecular weight of β-lactoglobulin A at pH 4.6, 16°, plotted against total protein concentration, fall on a smooth curve, even though the values were obtained in several experiments performed with different initial concentrations. This provides indirect evidence that the volume change is insignificant (Howlett *et al.*, 1970, 1972). Nevertheless, in the absence of a precise value of ΔV, it was decided to investigate the effect of deuterium oxide by methods which did not involve the generation of a pressure gradient.

(ii) Frontal gel chromatography studies. Figure (II-5) presents the elution profile obtained in the frontal analysis of β-lactoglobulin A in deuterium oxide, pD 4.65, I = 0.1 at 12°. It was noted earlier in this Chapter that theory for the mass migration of a rapidly equilibrating dimer-octamer system predicts, in the absence of boundary spreading, a hypersharp boundary on one side, and a spread reaction boundary with a point of inflection (corresponding to a minimum in the first derivative plot) on the other. The result in Figure (II-5) is in qualitative agreement with this description except for the point of inflection on the trailing side which has been masked by axial dispersion.

By means of equation (II-25), the elution volume of the dimer, V_A, was calculated from the median bisector of the spread boundary, ($\bar{V}$ = 31.2 ml), the corresponding z-average elution volume (V_z = 31.9 ml), and the elution volume of excluded octamer V_C, which was found by adding the loading volume (17.0 ml) to the void volume (11.6 ml) of the column. The value of V_A obtained (39.6 ml), together with the values for $\bar{V}$ and V_C were then substituted in the chromatographic analogue of equation (II-22) to calculate log X in D_2O at 12°. The resulting value of 12.5 may be compared with that of 10.9 found by a similar analysis of the frontal analysis experiment conducted in aqueous acetate buffer pH 4.65, (I = 0.1) at 12°. The values for the relevant parameters in this experiment were $\bar{V}$ = 33.6 ml, V_z = 34.3 ml, V_C = 28.6 ml and V_A = 38.3 ml. A comparison of the two values of V_A, found in two experiments with columns of slightly different size, may be accomplished by transforming the data to a distribution coefficient K_{av} which is independent of column size (Porath, 1962). K_{av} was found to be 0.53 in the aqueous environment and 0.60 in deuterium oxide. Since V_A is a derived quantity, the agreement is reasonable, and provides a first indication that the size and shape properties of the dimer of β-lactoglobulin A are not markedly affected by the change of environment. This was subsequently confirmed by measuring the optical rotatory dispersion of 0.01% solutions at 20° (conditions under which the protein is almost entirely dissociated into dimers in either medium) in the ultra violet region, 220-260 nm. The spectrum of

the protein in deuterium oxide buffer was identical to that obtained in the aqueous buffer. Of greater interest though is the support which frontal analysis gives to the conclusion drawn from the sedimentation velocity experiments; that the extent of association of the protein is greater in the deuterium oxide medium.

(iii) Optical Rotatory Dispersion (ORD) studies. Clearly, ORD measurements do not involve mass migration, but their inclusion here is justified because they provide, in certain cases, a weight-average quantity which may be used to estimate values of X'. McKenzie *et al.* (1967) measured the optical rotation of β-lactoglobulin A in aqueous buffer, pH 4.6, I = 0.1 at several wavelengths, and at a number of different temperatures. For each temperature studied, the measurements were transformed and plotted according to the phenomenological equation of Moffitt and Yang (1956) (see also Chapter V) to yield straight line plots with ordinate intercept $\bar{a}_0$. These workers chose to consider $\bar{a}_0$ as a weight-average quantity

$$\bar{a}_0 = (c_A a_{0,A} + c_C a_{0,C})/\bar{c} \qquad \text{(II-32)}$$

where $a_{0,A}$ and $a_{0,C}$ are the hypothetical Moffitt-Yang parameters of pure A and C, respectively. There is no theoretical justification for this formulation; but, if it is correct, it follows by analogy to equation (II-22) that

$$X' = \bar{c}^{1-n} (a_{0,A} - a_{0,C})^{n-1} (a_{0,A} - \bar{a}_0)/(\bar{a}_0 - a_{0,C})^n \qquad \text{(II-33)}$$

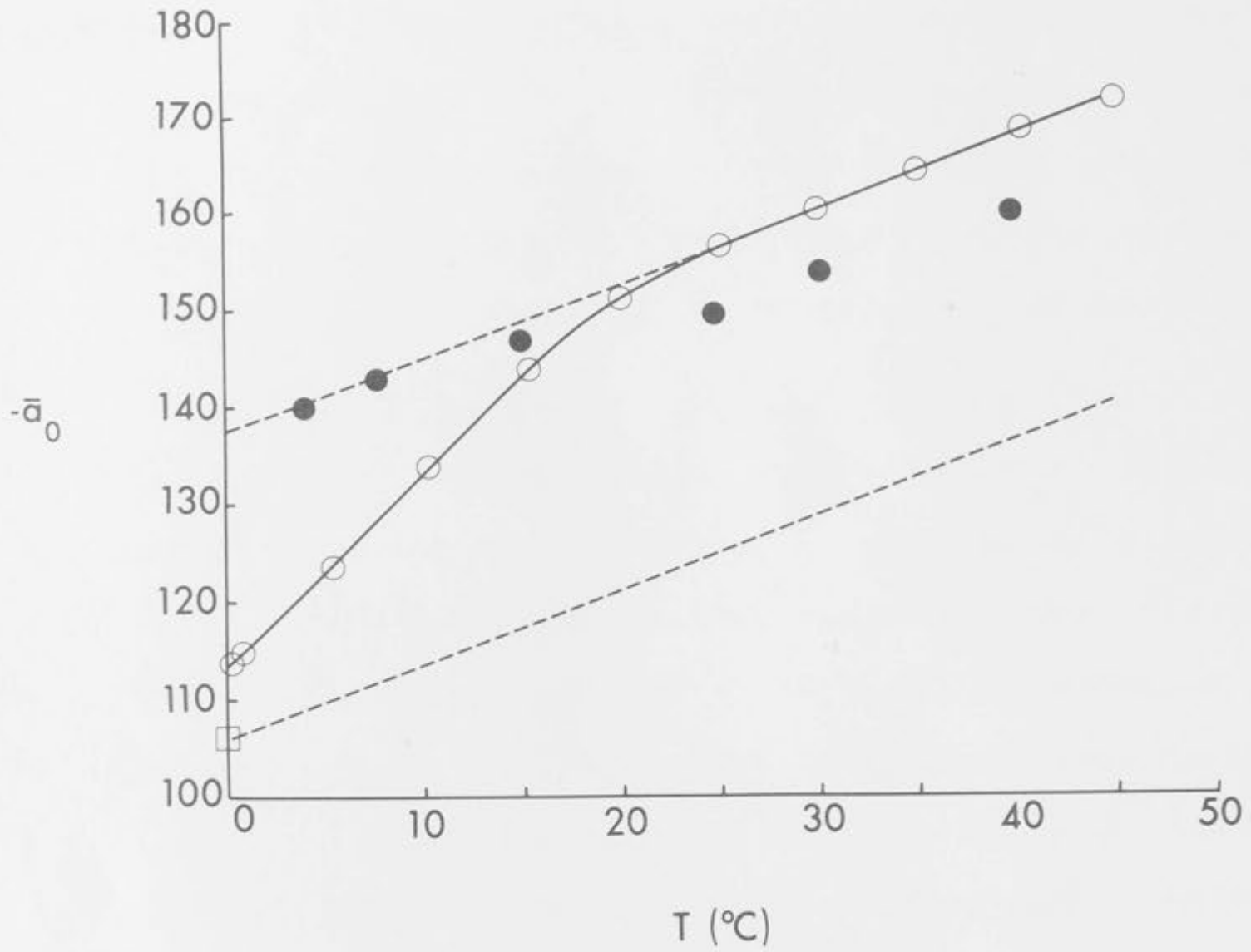


FIGURE II-6

The effect of temperature on the Moffit-Yang parameter, $\bar{a}_o$, of β-lactoglobulins A (O), and B (●) (13 g/l) in an aqueous solution of 0.1 M sodium acetate, 0.09 M acetic acid, pH 4.65. The intercept of the line representing the hypothetical contribution of octamer $a_{o,p}$ was found by determining $\bar{a}_o$ of a 100 g/l solution of β-lactoglobulin A (□).

McKenzie *et al.* (1967) used equation (II-33) to calculate X' at various temperatures, and hence a value of the enthalpy change. The reasonable agreement between these values and those described earlier from light scattering and Archibald weight-average molecular weight measurements justified the application of equations (II-32) and (II-33). On the other hand, it is not suggested that this formulation is necessarily valid for polymerising protein systems other than β-lactoglobulin A.

Figure (II-6) presents the temperature variation of $\bar{a}_0$ of a solution of β-lactoglobulin A (13 g/l) in the same aqueous buffer as that employed by McKenzie *et al.* (1967). On the basis that log X = 10 at 12° (Table II-1) and ΔH = -60 Kcal/mole (McKenzie *et al.*, 1967) it may be shown by means of equation (I-52) that there is only ~ 2% octamer in a solution of concentration 13 g/l at 25°. Thus the temperature dependence of $\bar{a}_0$ above 25° (Figure II-6) reflects, presumably, a changing environment of particular residues in the dimeric form alone. Extrapolation of this linear portion of the plot back to 0° gives hypothetical values of $a_{0,A}$ at lower temperatures. This procedure is not without criticism, but is vindicated by the finding that the extrapolated line is parallel to one obtained with β-lactoglobulin B which does not associate measurably over the entire temperature range. Hypothetical values for $a_{0,C}$ were also obtained by the procedure suggested by McKenzie *et al.* (1967). It was calculated that a 10% solution of β-lactoglobulin A at 0° would contain, essentially, only octamer. The experimentally obtained value of $\bar{a}_0$ of this solution was plotted in Figure (II-6),

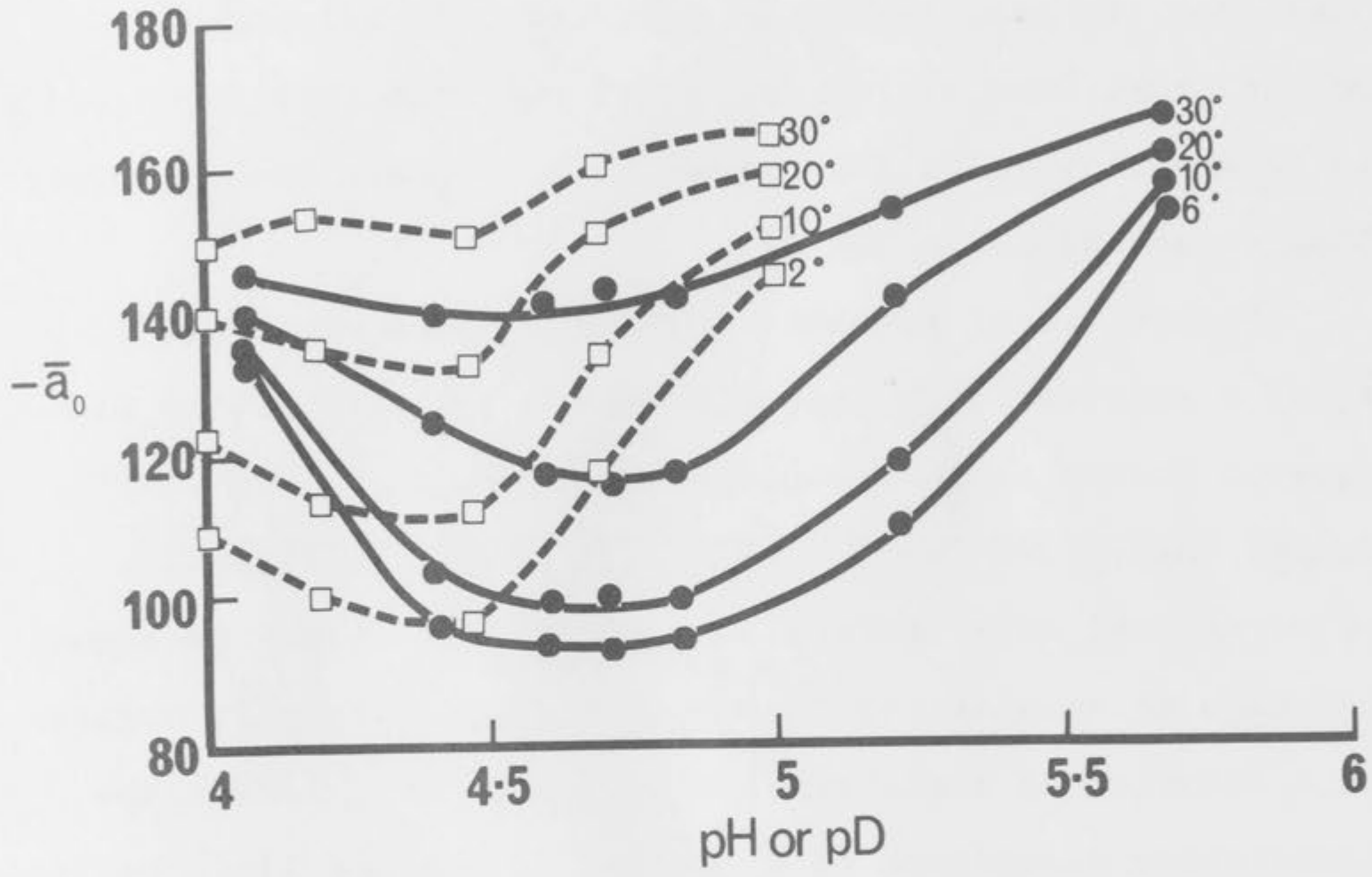


FIGURE II-7

The effect of temperature on the pH (or pD) dependence of the Moffitt-Yang parameter, $\bar{a}_o$, of β-lactoglobulin A (13 g/l). The solid curves refer to β-lactoglobulin A in D_2O acetate buffers and the broken curves to the protein in aqueous acetate buffers.

and with this point as origin, a line was drawn parallel to that describing the temperature dependence of $a_{0,A}$. The value of $\bar{a}_0$ at any temperature, together with the corresponding values of $a_{0,A}$ and $a_{0,C}$ read from Figure (II-6) were substituted into equation (II-33) to determine X'.

A similar procedure was used to investigate the effect of temperature on the association of β-lactoglobulin A in an aqueous acetate buffer of pH 4.4 and in a deuterium oxide buffer of pD 4.65.

Figure (II-7) presents $-\bar{a}_0$ values as a function of pH (or pD) at several different temperatures. The solid curves (referring to the D_2O medium) exhibit a broad minimum centred at pD 4.65 - 4.80, for all temperatures studied. It follows that the extent of association is maximal in this pD range and decreases with increasing temperature.

A similar conclusion is reached on examination of the broken curves in Figure (II-7) (referring to the aqueous medium) except that the minimum occurs at pH ~ 4.4, a result in basic agreement with that presented by McKenzie *et al.* (1967). Values for log X corresponding to these minima are plotted, in Figure (II-8), against the reciprocal of the absolute temperature at which each estimate was made. Those values obtained in the D_2O medium in the range pD 4.65 - 4.80 are reasonably grouped about a single straight line whose slope, determined by least squares regression, gives an estimate for the enthalpy change $\Delta H(D_2O)$ of -69 ± 2 Kcal/mole. The results in Figure (II-7) suggest that these values of log X should be compared with those obtained in the aqueous buffer at pH 4.4, where the apparent degree of association is maximal. It is clear from Figure (II-8) that X is

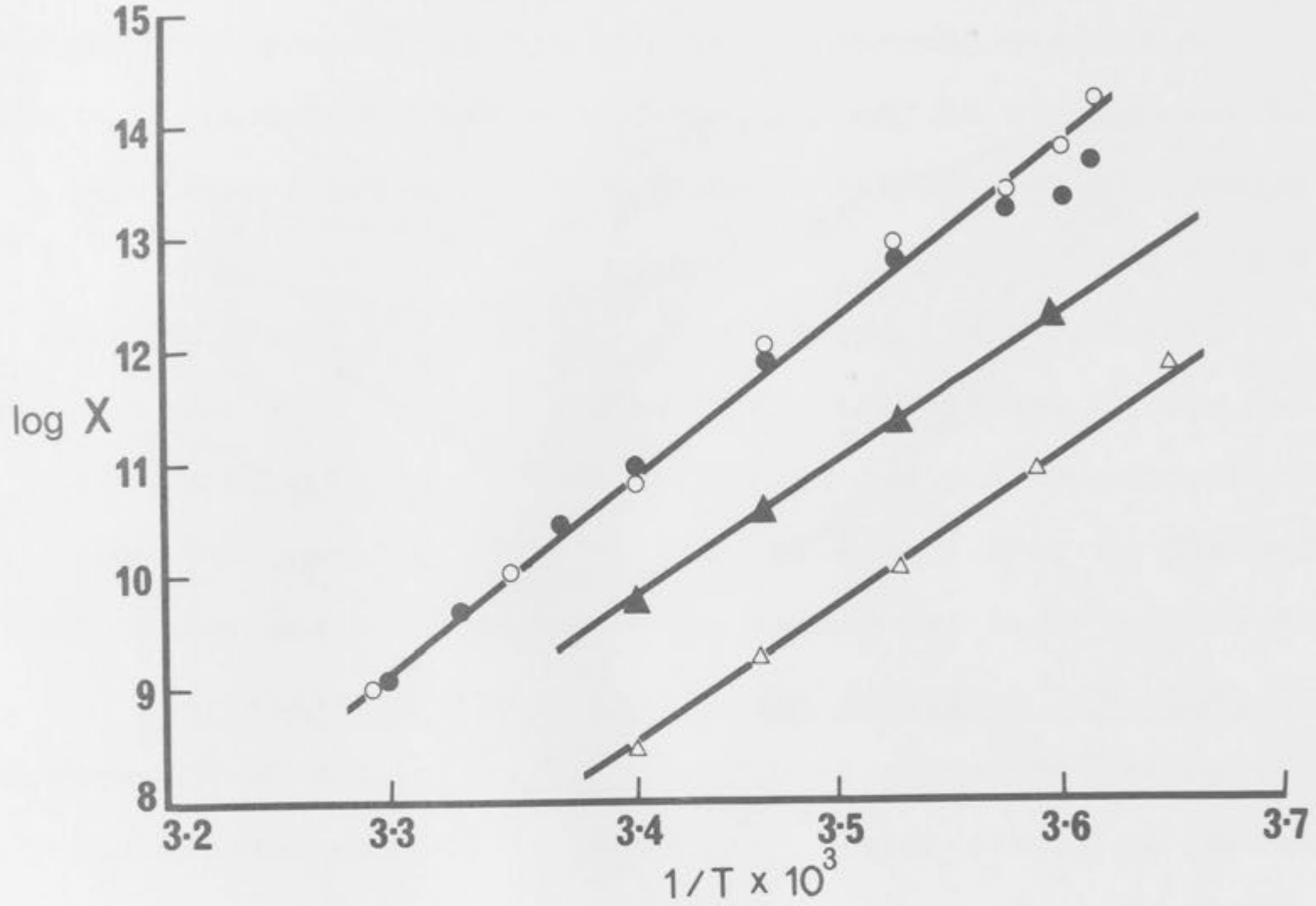


FIGURE II-8

A plot of log X values (derived from optical rotatory dispersion measurements) *vs.* the reciprocal of the absolute temperature. The solid lines were calculated by the method of least squares and the experimental points are as follows: ○, pD 4.65; ●, pD 4.80; △, pH 4.65; ▲, pH 4.47.

decreased at all temperatures in this aqueous medium. Since other methods (McKenzie *et al.*, 1967) have indicated that the maximum degree of association occurs near pH 4.6 in aqueous acetate buffer, the data obtained in this study at pH 4.6 (Figure II-6) have also been included in Figure (11-8). Regardless of which comparison is made, however, the basic conclusion that D_2O increases the extent of association remains unaltered. The enthalpy change for the association in both the aqueous environments was calculated to be -64 ± 2 Kcal/mole.

(c) Discussion

In order to compare the values of log X found by the four different methods employed in this study, all results obtained at 12° in deuterium oxide, pD 4.65 and water, pH 4.65, are collated in Table (II-2). The average value, 10.1, of the estimates for log X in the aqueous environment, is in excellent agreement with that (10.0) reported by McKenzie *et al.* (1967), and is significantly less than the average value (11.6) for the deuterium oxide medium. The table also illustrates the general finding that results obtained by treating the sedimentation coefficient as a weight-average quantity (equation (II-31)) were consistently low. This is almost certainly due to the necessity of correcting sedimentation coefficients to 20° in water using equation (II-30), which assumes a linear dependence of the coefficients on ratios of viscosity and buoyancy terms. Nevertheless it is clear, by all methods, that deuterium oxide enhances the association of β-lactoglobulin A. This conclusion remains valid

TABLE II-2

Values of log X for the dimer-octamer association of bovine β-lactoglobulin A at 12° in 0.1 M sodium acetate aqueous and deuterium oxide buffers $\{^{pH}_{pD}\ 4.65\}$

method of estimation	solvent	
	deuterium oxide	water
	pD 4.65	pH 4.65
sedimentation velocity (equation II-20)	11.3	10.0
sedimentation velocity (equation II-22)	10.0	9.6
frontal gel chromatography	12.5	10.9
optical rotatory dispersion	12.4	9.8
Average values	11.6	10.1

regardless of whether results are compared at identical values of pH and pD, or at values for which ORD measurements indicate maximal association (Figures II-7 and II-8).

The average values of log X reported in the Table, together with the observed enthalpy changes may be used to calculate the entropy changes for the association: -178 e.u. in water; -189 e.u. in deuterium oxide. These values must be regarded as approximate, due to the uncertainty in values of log X, but they illustrate clearly that octamer formation in D_2O is associated with enthalpy and entropy changes which are, at least as negative as the corresponding values found in water. If the sole action of D_2O was to increase the strength of hydrophobic interactions which may partly contribute to octamer formation, it would be expected that $\Delta S^{\circ}(D_2O) > \Delta S^{\circ}(H_2O)$, which was not observed. On the other hand, caution must be exercised in completely excluding the possibility that strengthening of hydrophobic interaction may contribute to the increase in association in deuterium oxide because observed enthalpy and entropy changes are the sums of such changes associated with each step in the association mechanism. Timasheff and Townend (1969) have proposed, for example, in their equation (4), a mechanism of octamer formation which involves protonation of the dimer unit together with a conformational change to a form which alone can octamerize via hydrogen (and possibly hydrophobic) bond formation. The conclusion, which can be drawn from the present study, is that deuterium oxide must exert its affect on at least one step in the mechanism not involving hydrophobic interaction. It is reasonable therefore to

comment on the possibility that the shift in the equilibrium induced by D_2O is a direct consequence of hydrogen-deuterium exchange, since it has been postulated that deuterium bonding is of greater strength than hydrogen bonding (Némethy and Scheraga, 1964). This isotope effect may operate in β-lactoglobulin A by strengthening an intermolecular hydrogen bond such as that postulated between aspartic and glutamic acid residues (Armstrong and McKenzie, 1967). Two further points concerning a possible hydrogen-deuterium exchange merit brief comment. First, it is clear from Figure (II-7) that the enhancement of association in D_2O is accompanied by a shift in the value of the pH (pD) corresponding to the minima, the difference being approximately 0.2 - 0.4 unit. The positions of these minima are determined partly by intermolecular forces (probably mainly of the hydrogen bonding type) and partly by net electrostatic repulsive forces. The pK of weak acids increases on transfer from H_2O to D_2O (Glascoe and Long, 1960; Bell, 1959), and, if the ionising groups on the protein behave similarly, this would explain (albeit qualitatively) the shift observed in Figure (II-7). Secondly, the rate of hydrogen-deuterium exchange varies markedly with the groups involved, but would be extremely rapid for external carboxyl groups (Hvidt and Nielson, 1966). Certainly the optical rotation of a solution of β-lactoglobulin A made by diluting 1 volume of 10% aqueous solution with 9 volumes of deuterium oxide reaches a stationary value in the time taken to fill a conventional ORD cell and place it in the polarimeter (approximately 3 minutes).

In a general context, these findings show that an increased association in deuterium oxide is not direct evidence for the sole operation of hydrophobic interactions in polymer formation (*cf.* Lee and Berns, 1968). Aune, Goldsmith and Timasheff (1971) who observed that the extent of association of α-chymotrypsin was increased in deuterium oxide also suggest that deuterium oxide may affect interactions which are not hydrophobic. On the other hand, it is not suggested that deuterium oxide is without effect on hydrophobic interactions, for indeed, the evidence is compelling (Lee and Berns, 1968). It is suggested, in view of the present results that it is unwise to advocate the use of D_2O as a discriminatory method for testing the relative importance of hydrophobic interactions.

This study of the effect of temperature and change of solvent on the polymerisation of β-lactoglobulin A is, of course, a restricted example of the much wider problem of studying acceptor self-interactions and the factors which affect them, but it has illustrated the use of mass migration techniques in approaching this type of problem. In comparing the extent of association in one environment with the extent of association in another, it has been necessary in this Chapter to perform two separate experiments in the different environments. In the next Chapter, the application of frontal gel chromatography is extended to permit a direct comparison of the extents of association of a protein in two different environments in a single experiment. Although β-lactoglobulin A is not known to be a biologically important acceptor system, it is again employed as a model system to

test the method prior to its application to the study of the action of effectors on haemoglobin.

CHAPTER III

DIFFERENTIAL CHROMATOGRAPHY

The studies on β-lactoglobulin in the last Chapter provided an example of the need to compare the extents of association of a protein in two different environments. In general this comparison may always be made by performing two separate experiments, but unless the difference between the values of the observable parameter in the two environments is large (for example, Figure II-4), the comparison requires the subtraction of two numbers which are of comparable magnitude and which are both subject to error. With separate experiments a confident comparison can be made only with a knowledge of the standard error, whose estimation requires that each experiment be repeated several times. There is an alternative possibility that an experiment may be designed to permit a direct comparison of the behaviour in the two environments, by difference. The differential sedimentation velocity technique of Hersh and Schachman (1955) in which interference optics are used to compare the sedimentation rates of two boundaries in separate channels of an ultracentrifuge cell, is one example of such a design which provides a direct difference. Direct comparisons of weight-average elution volumes are also possible in gel chromatographic experiments. This is the subject of the present Chapter which commences with a description of three means of conducting a differential chromatographic experiment.

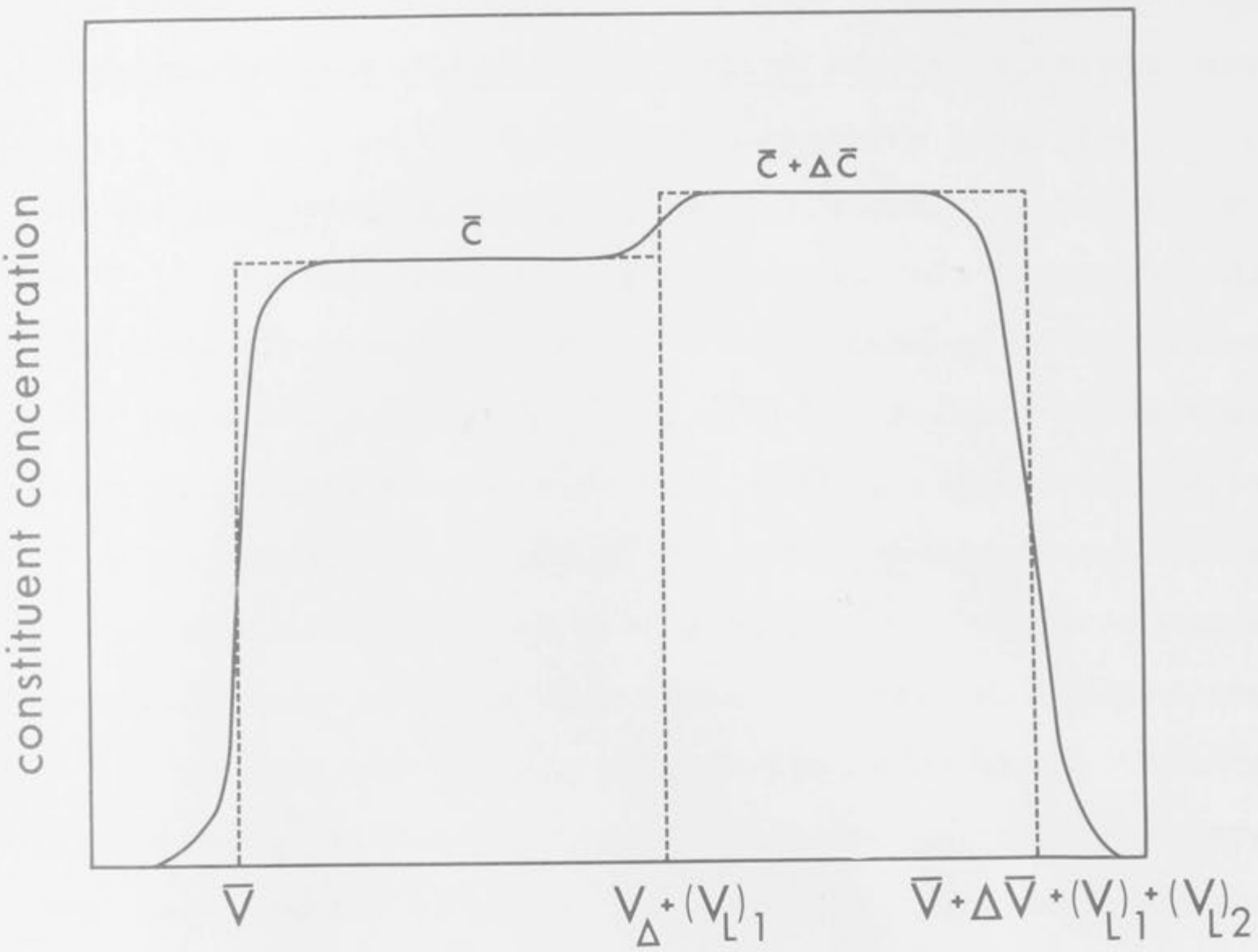


FIGURE III-1

A schematic diagram of the elution profile obtained by eluting a protein solution of concentration $\bar{c}$, with a second solution of the same protein in the same solvent but with a total concentration $\bar{c} + \Delta\bar{c}$. $(V_L)_1$ and $(V_L)_2$ are the loading volumes of the solutions of concentration $\bar{c}$ and $\bar{c} + \Delta\bar{c}$, respectively, and the median bisector of each boundary is indicated by a vertical line.

1. A description of the various types of differential chromatography.

(a) A single protein studied at two different concentrations in a fixed environment

The design originally proposed by Chiancone *et al.* (1968) involves the application of a protein solution of concentration $\bar{c}$ to a column pre-equilibrated with the same solvent. This solution is then eluted with a second solution of the same protein in the same solvent, but with a total concentration $\bar{c} + \Delta\bar{c}$. Figure (III-1) is a schematic diagram of the resultant elution profile, and it can be seen that sufficient volumes of the two solutions are used to ensure the occurrence of plateau regions of concentrations $\bar{c}$ and $\bar{c} + \Delta\bar{c}$. If these volumes are $(V_L)_1$ and $(V_L)_2$, respectively, the total amount (grams) of solute added is $\bar{c}(V_L)_1 + (\bar{c} + \Delta\bar{c})(V_L)_2$. For mass to be conserved, this amount must equal the sum of the areas of the rectangles described by the broken lines in Figure (III-1), the vertical lines being the median bisectors of each observable boundary.

$$\bar{c}(V_L)_1 + (\bar{c}+\Delta\bar{c})(V_L)_2 = \bar{c}(V_\Delta + (V_L)_1 - \bar{V}) + (\bar{c}+\Delta\bar{c})(\bar{V}+\Delta\bar{V}+(V_L)_2 - V_\Delta) \qquad \text{(III-1)}$$

Equation (III-1) may be rearranged to give

$$V_\Delta = \{(\bar{c}+\Delta\bar{c})(\bar{V}+\Delta\bar{V}) - \bar{c}\bar{V}\}/\Delta\bar{c} \qquad \text{(III-2)}$$

from which it can be seen that

$$\lim_{\Delta\bar{c}\to 0} V_\Delta = d(\bar{V}\bar{c})/d\bar{c} \qquad \text{(III-3)}$$

Equation (III-3) is equation (5) of Chiancone *et al.* (1968) who drew attention to its similarity to the equation of Miller (1909) for a difference boundary in electrophoresis, and to the equation of Hersh and Schachman (1955) for a difference boundary in sedimentation. In Chapter II it was pointed out that elution volumes could directly replace velocity terms in the equations for mass migration to give the analogous equations for gel chromatography. Replacing $\bar{V}$ in equation (III-3) with $\bar{v}$ yields

$$\lim_{\Delta\bar{c}\to 0} V_{\Delta} = d(\bar{v}\bar{c})/d\bar{c} = \bar{u} \qquad \text{(III-4)}$$

which is clearly the fundamental mass balance equation describing diffusion free migration (equation II-9). This type of experiment, therefore, repeated for various values of $\bar{c}$, provides a means of constructing a diffusion-free profile of $\bar{u}$ *vs.* $\bar{c}$. Furthermore, if the solute self-interacts according to the scheme $nA \rightleftharpoons C$, the data may be used in conjunction with equation (II-16) to evaluate the equilibrium constant X', although a prior knowledge of n, v_A and v_C is again required.

(b) <u>Two different proteins studied at the same concentration in a fixed environment (Gilbert, 1966b)</u>

The experiment involves the elution of one protein, A, from a column, with a solution of a second, different protein B, whose plateau concentration is identical to the first. The two proteins must migrate with their respective weight-average velocities corresponding to the weight-average elution volumes $\bar{V}_A$ and $\bar{V}_B$. If both proteins A and B are self-interacting according to the schemes $nA \rightleftharpoons C$ and $mB \rightleftharpoons D$,

a spread reaction boundary on the trailing side of the elution profile of A is followed by a hypersharp advancing front of B. This interesting situation is illustrated in Figure (III-2) adapted from Nichol and Winzor (1972). The median bisector of the trailing side is constructed as the vertical broken line DCB, and the area BDEG = $(\bar{V}_B - \bar{V}_A)\bar{c}$ is related to the experimentally obtainable area in the following way,

$$\begin{aligned} \text{area BDEG} &= \text{area BCFG} + \text{area DCFE} \\ &= \text{area BCFG} + \text{area DCJ} - \text{area EFJ} \\ &= \text{area BCFG} + \text{area ACB} - \text{area EFJ} \\ &= \text{area AFG} - \text{area GHI} \end{aligned}$$

Thus, the difference between the areas of the observed 'dip' and 'hump' equals $\bar{c}(\bar{V}_B - \bar{V}_A)$, and provides a direct measure of the difference between the weight-average elution volumes, since $\bar{c}$ is chosen at the discretion of the experimenter. In turn, this difference may be related to the degrees of association of the two proteins.

Let $\alpha_A (= c_C/\bar{c})$ and $\alpha_B (= c_D/\bar{c})$ be the degrees of association of A and B respectively. Then by equation (II-8),

$$\bar{V}_B - \bar{V}_A = V_B(1-\alpha_B) + V_D\alpha_B - V_A(1-\alpha_A) - V_C\alpha_A \qquad \text{(III-5)}$$

Equation (III-5) may be simplified in cases in which, for example, A and B are genetic variants of the same protein because it is likely that $V_A = V_B$, $V_C = V_D$ and hence

$$\bar{V}_B - \bar{V}_A = (\alpha_B - \alpha_A)(V_C - V_A) \qquad \text{(III-6)}$$

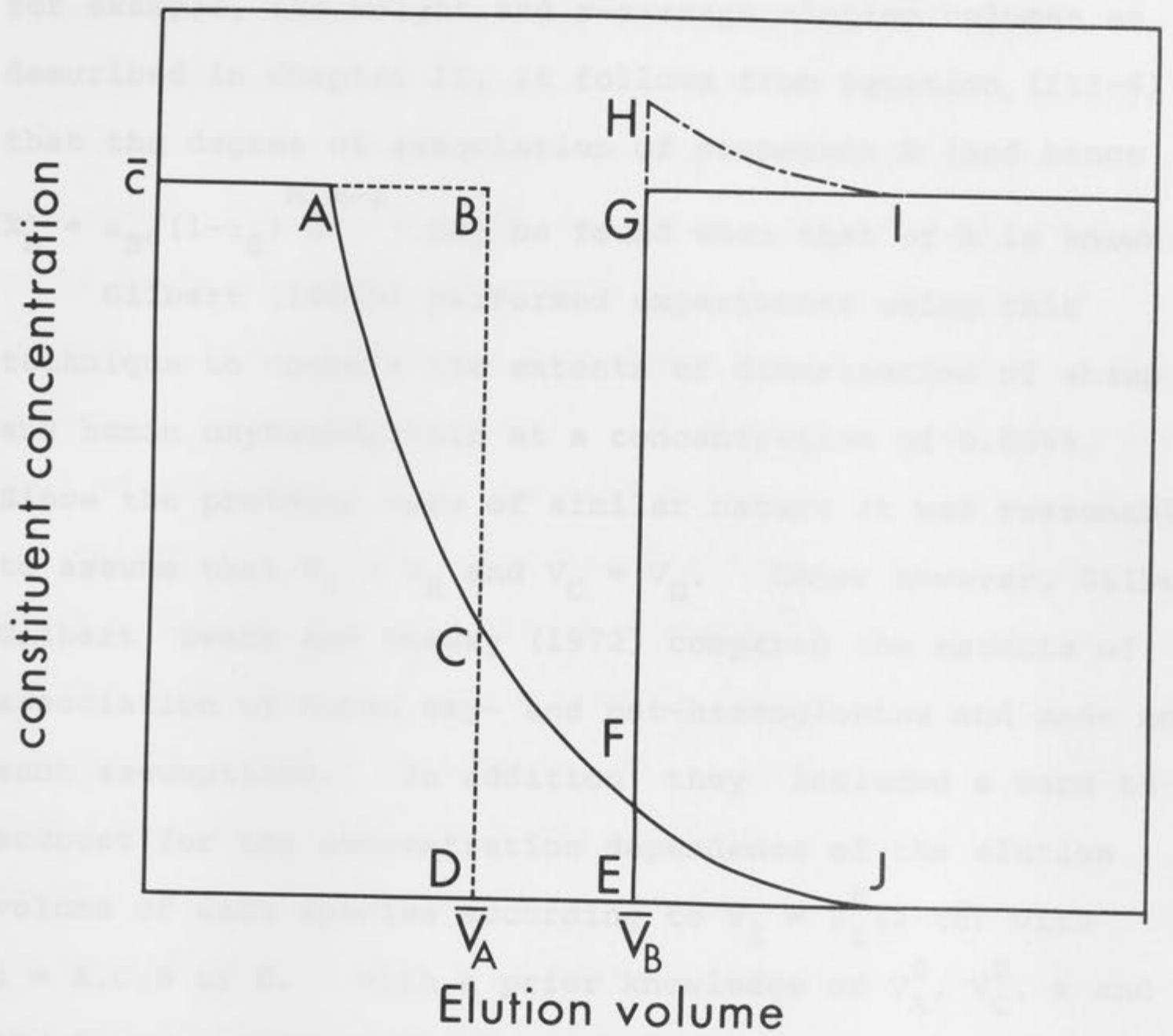


FIGURE III-2

Part of a theoretical elution profile resulting from the elution of a self-interacting protein, A, from a column, with a solution of a second, different protein, B, whose plateau concentration is identical to the first. The figure shows the trailing side of the elution profile of A followed by the hypersharp, advancing front of B. The vertical broken line DCB locates the median bisector of the trailing reaction boundary. Adapted from Nichol and Winzor (1972).

If the elution volumes V_A and V_C can be determined using, for example, the weight and z-average elution volumes as described in Chapter II, it follows from equation (III-6) that the degree of association of component B (and hence $X'_B = \alpha_B/(1-\alpha_B)^{m}\bar{c}^{m-1}$) may be found when that of A is known.

Gilbert (1966b) performed experiments using this technique to compare the extents of dimerisation of sheep and human oxyhaemoglobin at a concentration of 0.008%. Since the proteins were of similar nature it was reasonable to assume that $V_A = V_B$ and $V_C = V_D$. Later however, Gilbert, Gilbert, Owens and Shawky (1972) compared the extents of association of human oxy- and met-haemoglobins and made no such assumptions. In addition, they included a term to account for the concentration dependence of the elution volume of each species according to $V_i = V_i^0(1-k\bar{c})$ with i = A,C,B or D. With a prior knowledge of V_A^0, V_C^0, k and the dissociation constant of the oxy-haemoglobin, X'_A, a non-linear least squares regression analysis was employed to obtain estimates of V_B^0, V_D^0 and X'_B which are related by the parametric equations

$$\bar{V}_A-\bar{V}_B = \{(1-k\bar{c})/\bar{c}\}\{c_A V_A^0 + X'_A c_A^2 V_C^0 - c_B V_B^0 - X'_B c_B^2 V_D^0\} \quad \text{(III-7a)}$$

$$X'_A = c_C/c_A^2 \quad \text{(III-7b)}$$

$$X'_B = c_D/c_B^2 \quad \text{(III-7c)}$$

(c) <u>A single protein studied in two different environments</u>

The third type of differential chromatography to be discussed involves the application of a protein in one solvent

environment to a column pre-equilibrated with a different solvent environment. If the conformations or extents of association of the protein in the two environments differ to the extent that their weight-average elution volumes are also different, then, in principle, these weight-average quantities ought to be directly comparable in a single experiment. Gilbert (1966b) suggested experiments of this design without exploring their potentiality. In a general context, this type of experiment could be used to transfer a solute from one solvent to another (for example from water to deuterium oxide - Chapter II), to effect a change of ionic strength or pH, and finally, to study a protein in the presence and absence of a modifier (Chapter I). It is this final design of differential chromatography which is relevant to the present study. The theoretical aspects of the design are now described together with results obtained with bovine β-lactoglobulin A, and human haemoglobin.

2. Experimental results obtained using differential chromatography.

(a) Studies with bovine β-lactoglobulin A

The principal features of an experiment involving a change of environment are illustrated by the following results. 35.1 ml of β-lactoglobulin A (9.81 g/l) dissolved in a 0.1 ionic strength buffer, pH 4.6 (0.1 M sodium acetate, 0.09 M acetic acid) were added to a 1.3 x 23.8 cm column of Sephadex G-75 thermostatted at 4° and equilibrated with a 0.5 ionic strength buffer of the same pH (0.1 M sodium acetate, 0.09 M acetic acid, 0.4 M NaCl). The protein was then eluted with the 0.1 ionic strength buffer, the eluate being monitored spectrophotometrically at 280 nm. It is

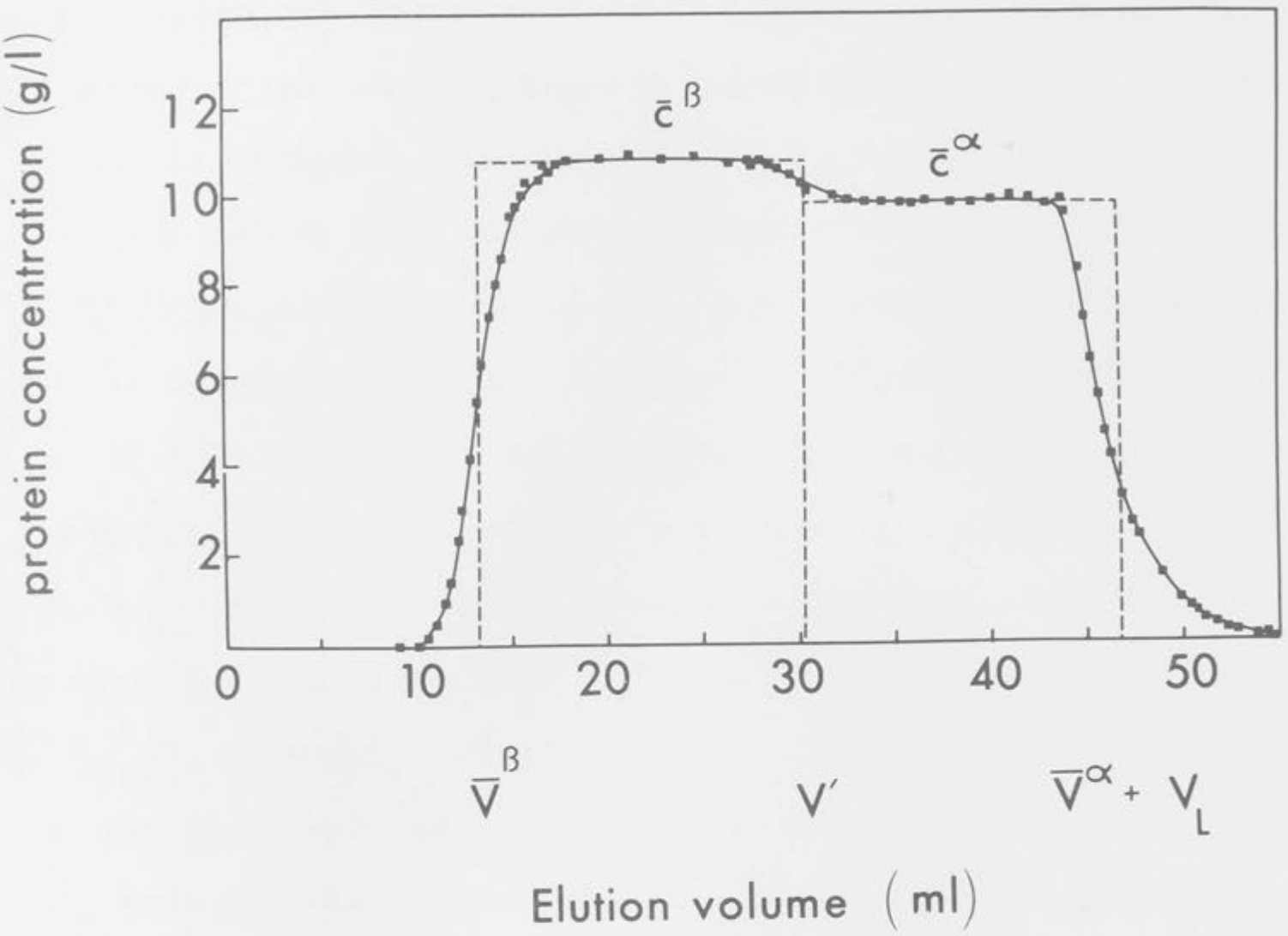


FIGURE III-3

The elution profile resulting from the application of 35.1 ml of β-lactoglobin A (9.81 g/l) in 0.1 I acetate, pH 4.65, to a 38 cm x 1 cm column of Sephadex G-75 equilibrated with 0.5 I acetate-chloride buffer (pH 4.65) at 4°. The flow rate was 0.5 ml/minute.

noted that the experimental procedure differs from that used by Gilbert (1966b) inasmuch that prior equilibration of the column with protein is not required. In the present design the plateau of protein in the ionic strength 0.5 medium is created during the experiment because of its faster migration rate compared with that of the sodium and chloride ions. In the profile of protein concentration (——) shown in Figure (III-3) there are two distinct plateau regions, the initial elution of protein being at a concentration greater than that applied, ($\bar{c}^{\alpha}$). The difference between the two observed plateau concentrations reflects a slower migration rate (larger weight-average elution volume) of β-lactoglobulin A in the environment with a higher ionic strength (β-phase). This result is consistent with the following geometric interpretation. The dotted lines in Figure (III-3) are median bisectors of observed concentration gradients and they permit the representation of irregular outlines as rectangles of equivalent area. If mass is to be conserved, the total amount of solute added to the column, $\bar{c}^{\alpha}V_L$, where V_L is the loading volume, must equal the sum of the rectangles shown in Figure (III-3). Thus

$$\bar{c}^{\alpha}V_L = (V'-\bar{V}^{\beta})\bar{c}^{\beta} + (\bar{V}^{\alpha}+V_L-V')\bar{c}^{\alpha} \qquad \text{(III-8)}$$

which yields, on rearrangement,

$$\bar{c}^{\alpha}/\bar{c}^{\beta} = (V'-\bar{V}^{\beta})/(V'-\bar{V}^{\alpha}) \qquad \text{(III-9)}$$

Equation (III-9) is the Johnston-Ogston equation (1946)* with V' identified as the elution volume of the median

FOOTNOTE

---

* The Johnston-Ogston equation was originally formulated to account for frictional interactions encountered in the sedimentation velocity of a mixture of two solutes. Later, Nichol and Ogston (1965b) stressed its applicability to the mass migration of a system in which constituent velocities were composition-dependent, regardless of the nature of the interactions leading to these dependencies. The above geometrical argument resulting in equation (III-9) provides a simple derivation of the general Johnston-Ogston equation and, moreover, illustrates the transformation from velocity terms to elution volumes (see Chapter II).

---

bisector of the protein constituent gradient between the α and β phases.

With reference to Figure (III-3) the values of the parameters in equation (III-9) are given by $\bar{c}^{\alpha}$ = 9.81 g/l, V' = 30.5 ml, $\bar{V}^{\beta}$ = 13.5 ml and $\bar{V}^{\alpha}$ = 11.5 ml. Their substitution in equation (III-9) yields a value of 10.96 g/l for $\bar{c}^{\beta}$, in close agreement with that observed experimentally. It was noted in the previous Chapter that β-lactoglobulin A exists, at pH 4.65, I = 0.1, as a mixture of essentially dimer A and octamer C. At 4° this association is characterised by an equilibrium constant X', of $1.08 \times 10^{-2}\ l^3.g^{-3}$ (Table II-1). The definitions of the equilibrium constant and weight-average elution volume yield the following simultaneous equations:

$$X'(c_A^{\alpha})^4 + c_A^{\alpha} - \bar{c}^{\alpha} = 0 \qquad \text{(III-10)}$$

$$\bar{V}^{\alpha}\bar{c}^{\alpha} = V_A c_A^{\alpha} + V_C(\bar{c}^{\alpha} - c_A^{\alpha}) \qquad \text{(III-11)}$$

where c_A^{α} is the equilibrium concentration of monomer in the α-phase and V_A and V_C are the elution volumes of the dimer and octamer species, respectively. With V_C taken as the void volume of the column (9.3 ml), equations (III-10) and (III-11) were solved for the remaining unknown, V_A = 13.9 ml. Equations (III-10) and (III-11), written for the β phase were then used to calculate c_A^{β} and hence X' for the 0.5 I environment, assuming V_A and V_C had the same values in both phases. A value of $1.0 \times 10^{-4}\ l^3.g^{-3}$ was found for X' in the 0.5 I medium which shows clearly that an increase in ionic strength promotes the dissociation of octameric

β-lactoglobulin A. This is in agreement with the unpublished findings (Howlett, 1972) which showed that the apparent weight-average molecular weight of β-lactoglobulin A decreased with increasing ionic strength. While a variation in ionic strength was used to illustrate the potentiality of differential chromatography in detecting perturbations of polymerisation equilibria it is clear that the effects of other variables could be studied in the same manner. Of particular interest in relation to the findings presented in Chapter II is the influence of an exchange of solvent on the extent of association of β-lactoglobulin A. A solution of this protein in a deuterium oxide medium pD 4.6, I = 0.1, was applied to a column (38 x 1 cm) equilibrated with aqueous acetate buffer pH 4.6, I = 0.1 at 12°. Two plateaux were again evident in the elution profile. The concentration gradient separating the two plateaux occurred at an elution volume V' = 33.0 ml which also corresponded to the median bisector of the emerging D_2O gradient measured separately with a pH meter. The initial elution of protein in the aqueous phase occurred with a weight-average elution volume, $\bar{V}(H_2O)$ = 15.5 ml corresponding to a plateau concentration of $\bar{c}(H_2O)$ = 7.57 g/l, while the concentration in the second plateau ($V > V'$) was equal to the applied concentration of 7.40 g/l and was associated with a weight-average elution volume of 52.0 ml, which on subtracting the loading volume, 36.9 ml, gives $\bar{V}(D_2O)$ = 15.1 ml. This behaviour is again consistent with equation (III-9). Thus the concentration ratio calculated from the right-hand side of equation (III-9) was 0.978, in excellent agreement with the value (0.977) measured spectrophotometrically and

by the method of Lowry, Rosebrough, Farr and Randall (1951). The difference between $\bar{V}(H_2O)$ and $\bar{V}(D_2O)$, confirmed by the existence of two plateaux indicates in a single experiment the greater proportion of octamer in the D_2O environment, a finding in complete accord with the basic conclusion drawn in Chapter II.

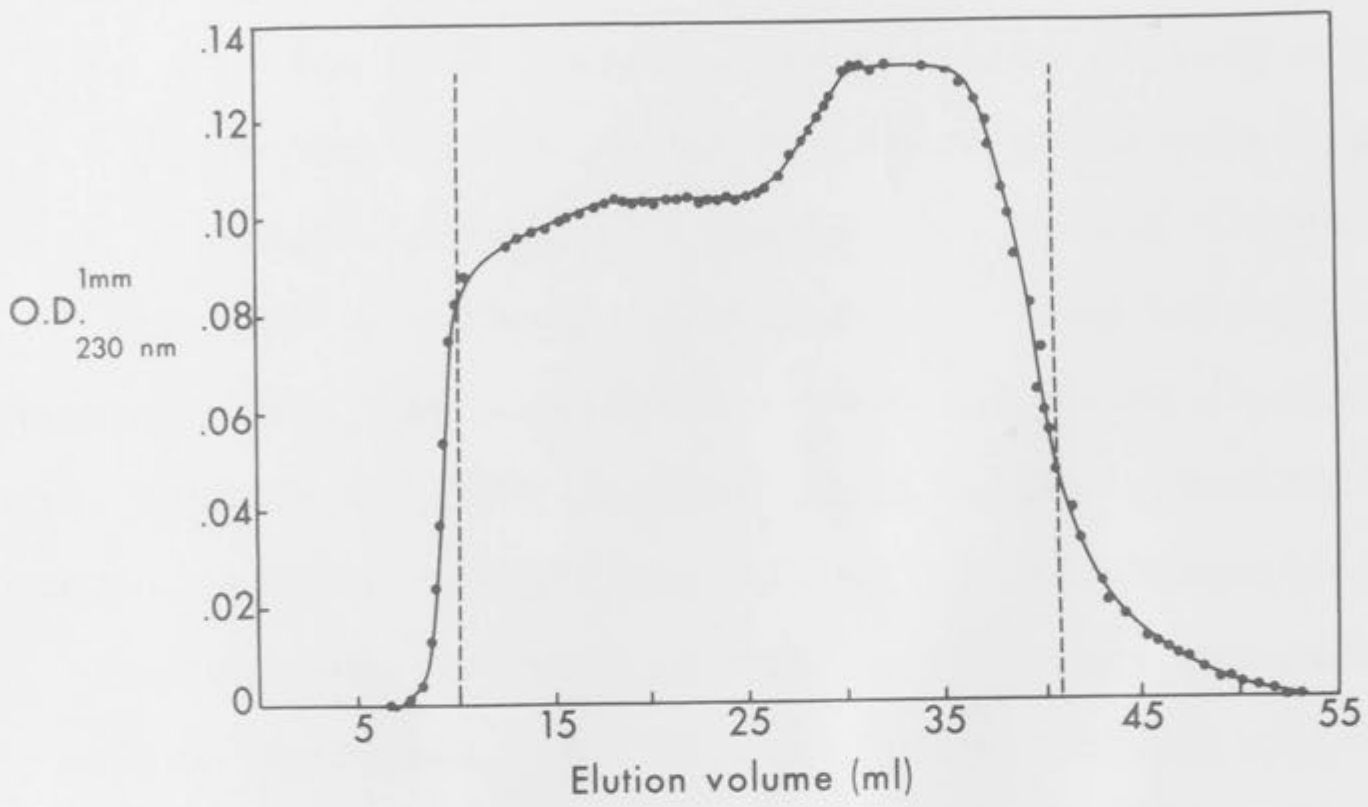
While the study of polymerising protein systems by differential chromatography requires that the elution volumes V_A and V_C using the chosen stationary phase, be different, the origin of this difference in no way affects the validity of equation (III-9). Thus the elution volumes of monomer and polymer may differ as a consequence of changes in shape as well as size. The applicability of the technique to the study of interacting systems involving only a shape change (isomerisation) is discussed in the next section.

(b) Studies with isomerising systems

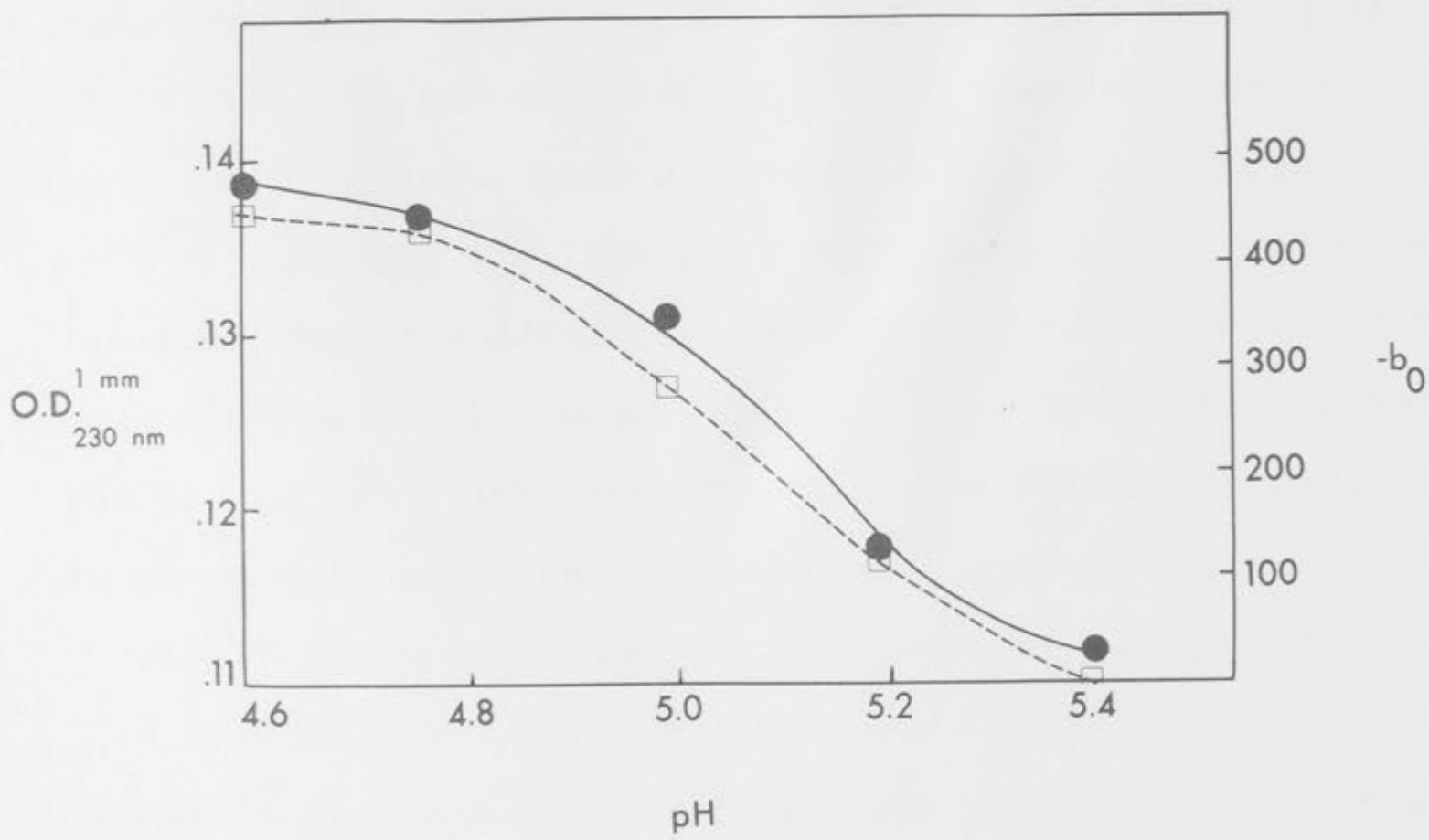
Between pH 4.6 and pH 2, bovine serum albumin undergoes an acid expansion (Bro, Singer and Sturtevant, 1955) which is sufficient to affect the velocity (and hence the elution volume) of the protein on Sephadex G-100. This conformational change is reflected in the occurrence of two plateaux in the elution profile when a solution of bovine serum albumin dissolved in an acetate buffer, ionic strength 0.1, pH 4.6 is applied to a column of Sephadex G-100 pre-equilibrated with a buffer of the same ionic strength, but of pH 2 (Figure 1a of Baghurst, Nichol, Richards and Winzor, 1972). Since this experiment was performed by Drs. R. J. Richards and D. J. Winzor, no further comment will be made here, except to note that the observed elution

volumes and plateau concentrations obeyed equation (III-9) exactly.

Another pH induced conformational change which might be studied chromatographically is the helix-coil transition of several synthetic polypeptides. Figure (III-4a) presents an elution profile obtained by loading a 0.1% solution of poly-L-glutamic acid, dissolved in an acetate buffer pH 4.8, ionic strength 0.2 on to a column of Sephadex G-200 pre-equilibrated with another acetate buffer of the same ionic strength but of pH 5.4. At first sight, the appearance of two distinct plateaux might suggest that the helical form of the polypeptide in the pH 4.8 environment moves much more slowly than the random coil form in a pH 5.4 environment. However, application of equation (III-9) shows that this interpretation, while intuitively reasonable, is incorrect. The elution volumes in the two environments, $\bar{V}$ (pH 5.4) = 10.0 ml, $\bar{V}$ (pH 4.8) = 10.2 ml are very close and they predict, according to equation (III-9), a ratio of plateau concentrations of 0.989, which contrasts with the observed ratio of 0.103/0.130 = 0.792. The origin of the difference in absorbance in the two environments was investigated and found to be due to a dependence of the molar extinction of the polypeptide at 230 nm on the helical content (Glazer and Smith, 1960). Figure (III-4b) shows the variation (——) of the optical density at 230 nm of a 0.1% solution of poly-L-glutamic acid with pH, and the corresponding variation (---) of the Moffitt-Yang parameter b_0 which is proportional to helical content (Urnes and Doty, 1961; Chen and Yang, 1971). Clearly the optical density is closely correlated with helical content, and the observed


.14
.12
.10
.08
.06
.04
.02
0
O.D. 1mm 230 nm
5
15
25
35
45
55
Elution volume (ml)

(a)


.14
.13
.12
.11
O.D. 1 mm 230 nm
500
400
300
200
100
$-b_0$
4.6
4.8
5.0
5.2
5.4
pH

(b)

FIGURE III-4

(a) The elution profile obtained by loading 30.5 ml of poly-L-glutamic acid (10 g/l) in 0.2 I acetate, pH 4.8, on to a 28.2 cm x 1.3 cm column of Sephadex G-200 pre-equilibrated with a 0.2 I acetate buffer of pH 5.4 at 20°. The profile is corrected for the difference in blank absorptions of the two buffers which was due to the higher total acetate content of the pH 4.8 buffer. The flow rate was 0.1 ml/minute.

(b) The pH dependence of the optical density (___) and the Moffitt-Yang parameter, b_o, (---) of poly-L-glutamic acid (10 g/l) in 0.2 I acetate.

variation accounts for the difference between the absorbances recorded for the two environments in Figure (III-4a). It could be noted that the failure of the present experiments does not exclude the possibility of studying helix-coil transitions and other conformational changes by chromatographic methods provided suitable stationary phases can be found. The experiment summarised in Figure (III-4a) does highlight the necessity of investigating the effect of environmental parameters on the assay procedure (albeit spectrophotometric or otherwise).

(c) The effect of modifiers: the haemoglobin-ATP system

(i) Theoretical considerations. It was pointed out in Chapter I that the slope of a binding curve obtained with a self-interacting acceptor may be markedly altered by the presence of a modifier which binds preferentially to one form (isomeric or polymeric) of the acceptor. This section is concerned with the theoretical aspects of a chromatographic technique for determining whether a modifier binds preferentially, and, if so, to which form of the acceptor. In an experiment in which an equilibrium mixture of acceptor and modifier is applied to a column pre-equilibrated with solvent alone, the acceptor will migrate faster than the modifier, and a differential experiment of the third design is established. This permits a direct comparison of the migration rates of acceptor in the presence and absence of the modifier. Such a design might prove useful for investigating systems in which the modifier induces a shape change in the acceptor as well as those in which it disturbs a pre-existing equilibrium of the type $nA \rightleftharpoons C$. In a practical vein, size changes ($n > 1$) are probably more

readily studied but the detailed discussion which follows is considerably simplified by focussing attention on systems in which n = 1. The experiment is performed by loading a large volume of a solution consisting of an equilibrium mixture of unbound A, C, E and the complexes AE_i (i = 1,2,...,w) and CE_j (j = 1,2,...,y), on to a column pre-equilibrated with solvent containing neither protein nor modifier. It is required to determine the behaviour of this system on the column and hence the nature of the elution profile. From Chapter I the molar concentrations of all forms of A and C are given by

$$[\bar{A}] = [A](1+N_A[E])^w \qquad \text{(III-12)}$$

$$[\bar{C}] = [C](1+N_C[E])^y \qquad \text{(III-13)}$$

where it is assumed, for simplicity that E binds to equivalent and independent sites with intrinsic binding constants N_A and N_C on A and C respectively. An apparent self-association constant X* is defined, as in equation (I-54b), by

$$X^* = [\bar{C}]/[\bar{A}]^n = X(1+N_C[E])^y/(1+N_A[E])^{nw} \qquad \text{(III-14)}$$

from which the total weight concentration of acceptor may be written

$$\bar{c}_A = M_A[\bar{A}] + nM_AX^*[\bar{A}]^n \qquad \text{(III-15)}$$

The corresponding constituent velocity is given by

$$\bar{v}_A\bar{c}_A = M_Av_A[\bar{A}] + nM_Av_CX^*[\bar{A}]^n \qquad \text{(III-16)}$$

where it has been assumed that all v_{AE_i} equal v_A and all $v_{CE_j} = v_C$, which is reasonable in chromatography if E is small ($v_C > v_A >> v_E$; distances positive in the direction of migration). Equations (III-15) and (III-16) may be used to reformulate the continuity equation ($ud\bar{c}_A - d\bar{v}_C\bar{c}_A = 0$, Chapter II) as

$$\{u-v_A+n^2(u-v_C)X^*[\bar{A}]^{n-1}\}d[\bar{A}]+n(u-v_C)[\bar{A}]^n dX^* = 0 \qquad \text{(III-17)}$$

Equation (III-17) will now be examined for the two cases in which modifier binds preferentially and non-preferentially to one form of the acceptor. The latter situation arises when $y = nw$ and $N_A = N_C$, and as a consequence, equation (III-14) reduces to $X^* = X$, or $dX^* = 0$. Equation (III-17) then has two solutions, $d[\bar{A}] = 0$ and

$$u = \frac{v_A+n^2v_CX[\bar{A}]^{n-1}}{1+n^2X[\bar{A}]^{n-1}} \qquad \text{(III-18)}$$

It can be seen that values of u satisfying equation (III-18) must lie between v_A and v_C, which implies that outside these limits (on either the ascending or descending side) $d[\bar{A}] = 0$, and hence from equation (III-15) $d\bar{c}_A = 0$. Thus, for the case of non-preferential binding, an experimenter measuring the total concentration of acceptor in all its forms will observe no change in concentration across the plateau region separating the boundaries of the A constituent on the advancing and trailing sides.

An illustration of the type of migration pattern obtained for non-preferential binding is shown in Figure (III-5), constructed for the simplest case $n = 1$, $p = 1$, $q = 1$.

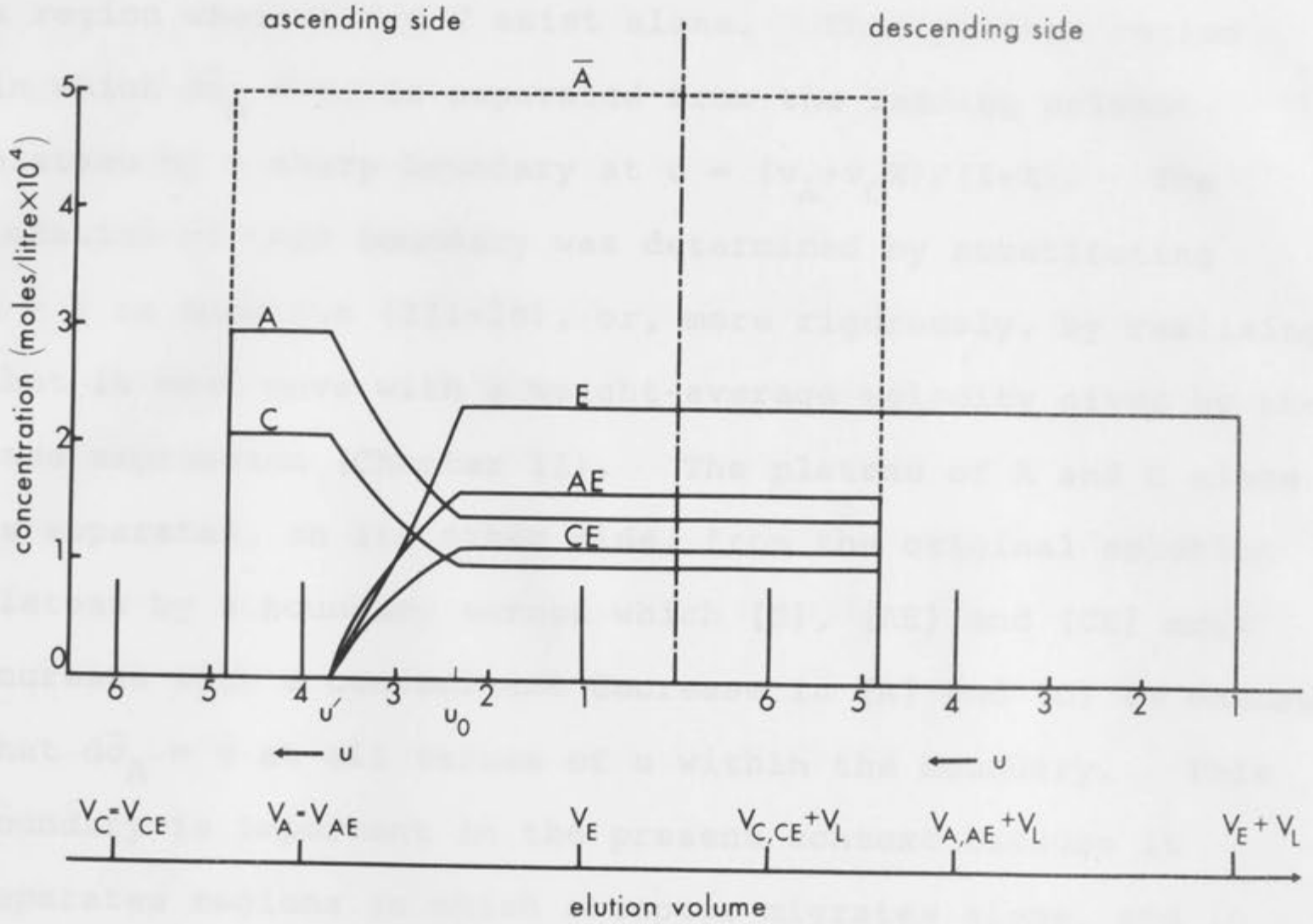


FIGURE III-5

A theoretical migration pattern of concentration plotted against the normalised distance parameter, u, for the system A⇌C with E binding with equal affinity to one site on A and one site on C.

Values for the isomerisation constant X and the binding constant N_A were taken to be 0.7, and 5000 l/g, respectively. The initial mixture was 0.0005 M in both total acceptor and effector, and the velocities of A, C and E were 6, 4 and 1 respectively.

On the advancing side, unbound acceptor species and complexes tend to migrate ahead of the modifier, but re-equilibration in order to satisfy the laws of mass action results in a region where A and C exist alone. This plateau region in which $d\bar{c}_A = 0$, is separated from the leading solvent plateau by a sharp boundary at $u = (v_A+v_CX)/(1+X)$. The location of this boundary was determined by substituting $n = 1$ in equation (III-18), or, more rigorously, by realising that it must move with a weight-average velocity given by the same expression (Chapter II). The plateau of A and C alone is separated, on its other side, from the original solution plateau by a boundary across which [E], [AE] and [CE] must increase with a concomitant decrease in [A] and [C] to ensure that $d\bar{c}_A = 0$ at all values of u within the boundary. This boundary is important in the present context because it separates regions in which acceptor migrates alone, and in the presence of effector (*cf.* Figures III-5 and III-3). It is not described by equation (III-18) since it lies outside the limits of v_A and v_C. The relevant expressions are derived in Appendix II from equation (III-17) and the continuity equation for effector. On the trailing side, the original solution plateau continues until the concentration of all forms of acceptor fall to zero. Since $d[\bar{A}] \neq 0$ over this boundary, it must occur at the unique value of u specified by equation (III-18). It is shown in Appendix I that $d[E] = 0$ at this value of u and thus a plateau of E alone continues at its initial equilibrium concentration until it is terminated by a sharp boundary at $u = v_E$. The essential features of Figure (III-5) have now

been described. With the appropriate alternative labelling of the abscissa it may be seen that the same figure also describes an elution profile. The broken line, representing the profile for total acceptor encloses an area which clearly equals $V_L\bar{c}_A$, confirming conservation of mass and emphasising that only a single plateau of acceptor will be observed for non-preferential binding.

The second situation in which the effector binds preferentially to one form of the acceptor will now be considered with reference to the particular case n = 1, p = 1, q = 0, selected for direct comparison with the result shown in Figure (III-5). For this system $X^* = X/(1+N_A[E])$ and dX^*, appearing in equation (III-17) is no longer zero but a function of [E]. Equation (III-17) becomes

$$(u-v_A)d[\bar{A}] + (u-v_C)d[C] = 0 \qquad \text{(III-19a)}$$

or $$(u-v_A)d[A] + (u-v_A)d[AE] + (u-v_C)d[C] = 0 \qquad \text{(III-19b)}$$

For the effector, whose total weight concentration is given by $\bar{c}_E = M_E\{[E]+[AE]\}$, the corresponding continuity equation is

$$(u-v_E)d[E] + (u-v_A)d[AE] = 0 \qquad \text{(III-20)}$$

In principle, equations (III-19) and (III-20) could be used, as before, to obtain a complete solution of the migration pattern. However, attention is focussed here on the behaviour of $\bar{c}_A$ as [E] increases from zero to its value in the original solution plateau on the advancing side. Subtracting equation (III-20) from (III-19b) and dividing

by $d[E] \neq 0$ yields

$$d[A]/d[E] = (u-v_E)/\{(u-v_A) + X(u-v_C)\} \qquad \text{(III-21)}$$

From equation (III-19a),

$$d[\bar{A}]/d[A] = -X(u-v_C)/(u-v_A) \qquad \text{(III-22)}$$

and multiplication of equations (III-21) and (III-22) gives

$$d[\bar{A}]/d[E] = -X(u-v_E)(u-v_C)/(u-v_A)\{(u-v_A)+X(u-v_C)\} \qquad \text{(III-23)}$$

Now, since $\bar{c}_A = M_A\{[\bar{A}]+[C]\}$, $d\bar{c}_A/d[E] = M_A\{d[\bar{A}]/d[E] + d[C]/d[E]\}$, and since $d[C]/d[E]$ is available from equation (III-21) as $d[C]/d[E] = Xd[A]/d[E]$, it can be seen that

$$d\bar{c}_A/d[E] = M_A X(u-v_E)(v_C-v_A)/(u-v_A)\{(u-v_A)+X(u-v_C)\} \qquad \text{(III-24)}$$

Since $v_E < u < v_A$ over this boundary, $(u-v_E) > 0$, $(u-v_A) < 0$, $(u-v_C) < 0$ and $(v_C-v_A) > 0$. Thus, from equation (III-24) $d\bar{c}_A$ and $d[E]$ are of the same sign. It is concluded that when E binds preferentially to the slower species A, two plateaux will be observed rather than one as depicted in Figure (III-5). The gradient in $\bar{c}_A$ separating the two plateaux will be of the same sign as the gradient in [E].

When E binds preferentially to the faster migrating species, it may be shown by a similar argument that there are, again, two plateaux, but $d\bar{c}_A/d[E]$ is negative. Thus, the existence of a gradient in $\bar{c}_A$ provides an indication of preferential binding while the sign of $d\bar{c}_A/d[E]$ indicates which acceptor species is preferred.

Although discussion has been limited, so far, to an isomerising acceptor, many of the conclusions drawn during this discussion are directly applicable to polymerising systems ($n > 1$). Whether binding is preferential or not, A and C must always emerge first, forming a hypersharp boundary moving with a weight-average velocity between v_A and v_C (Chapter II). When the binding is non-preferential, $d[\bar{A}] = 0$ for all u outside these limits and, accordingly, no concentration gradient is possible until $\bar{c}_A$ falls to zero on the trailing side. If mass is to be conserved, it follows that the median bisector of the latter boundary described by equation (III-18) must also move with a weight-average velocity corresponding to the original plateau concentration. The situation parallels that illustrated by the broken line in Figure (III-5) except that for $n > 1$ the trailing boundary will be spread between v_C and v_A. When binding is not preferential, the median bisector of the acceptor constituent gradient on the trailing side is related to X* (equations (III-14) and (III-17)) which is a function of X and [E], whereas the location of the hypersharp boundary on the advancing side, is a function of X only (given v_A and v_C). Hence, to conserve mass, a gradient in $\bar{c}_A (d\bar{c}_A/d[E] \neq 0)$ must arise between these two boundaries.

(ii) Preliminary results obtained with human haemoglobin and adenosine triphosphate (ATP). Normal adult haemoglobin is considered to exist near pH 5.5 as an equilibrium mixture of predominantly dimeric and tetrameric forms, the dimer consisting of dissimilar α and β polypeptide chains (Field and O'Brien, 1955; Chiancone and Gilbert, 1965;

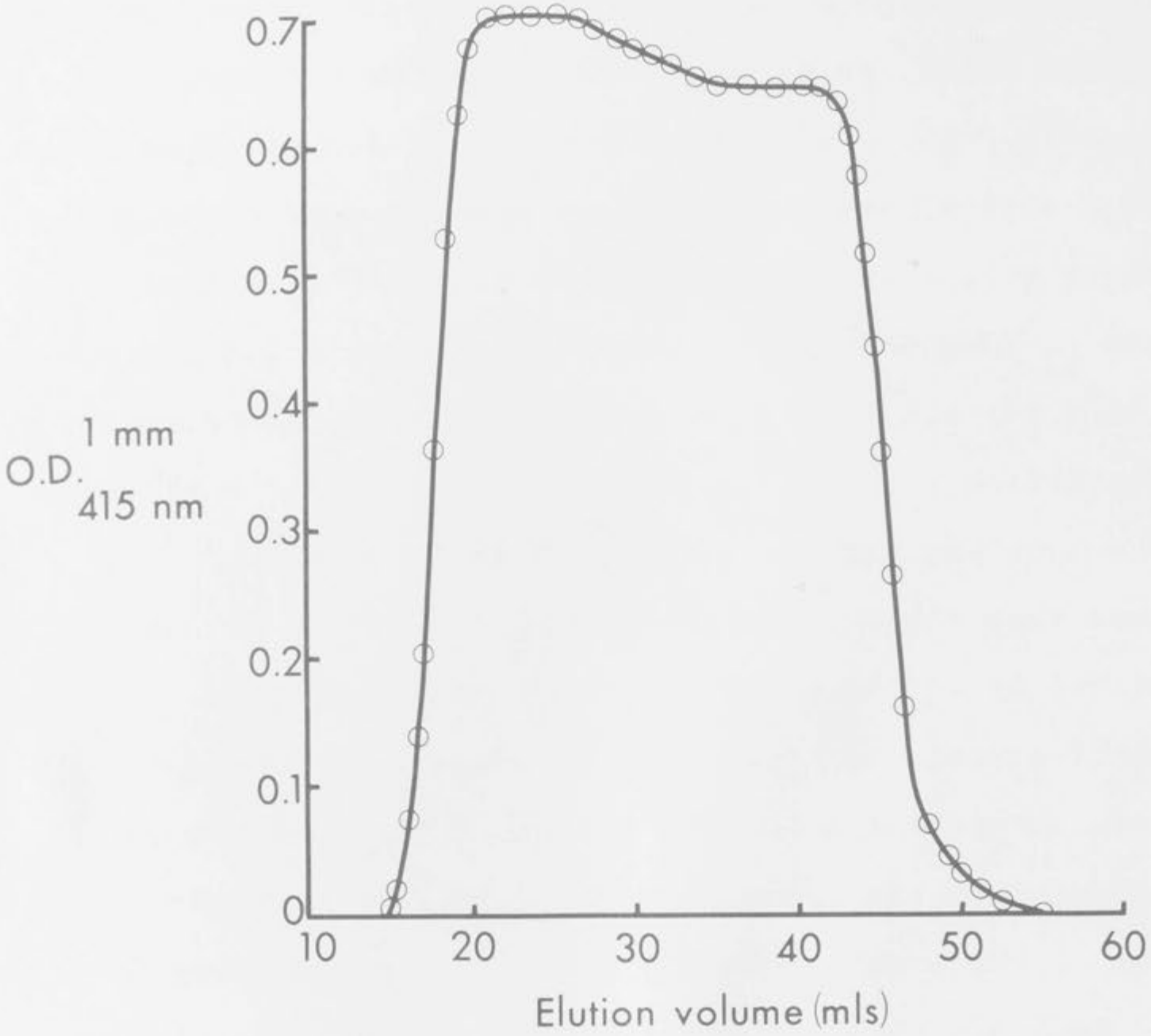


FIGURE III-6

The elution profile produced by application of 29.3 ml of pooled methaemoglobin (1 g/l) and adenosine-5'-triphosphate (0.75 g/l) in 0.25 M sodium acetate, pH 5.4 to a (26.3 cm x 1.3 cm) column of Sephadex G-75 equilibrated with the acetate buffer, pH 5.4 at 20°. The flow rate was 0.1 ml/minute.

Rossi-Fanelli, Antonini and Caputo, 1964). In a preliminary investigation of the effect of ATP on this equilibrium, a solution of 0.1% methaemoglobin and 0.075% ATP dissolved in 0.25 M sodium acetate, acetic acid, pH 5.4 was loaded on to a column of Sephadex G-75 equilibrated with the buffer only, and the eluate was monitored spectrophotometrically at 415 nm. The resultant elution profile is presented in Figure (III-6). Firstly, it is noted that the experimentally determined ratio of plateau concentrations $\bar{c}^{\alpha}$(ATP)/$\bar{c}^{\beta}$(no ATP) agrees closely with that predicted by equation (III-9), viz. 0.924, calculated with $\bar{v}^{\beta}$ = 17.6 ml, $\bar{v}^{\alpha}$ = 16.5 ml and V' = 31.0 ml. This provides an internal check that the concentration gradient separating the observed plateaux arises as a result of differential migration rather than a shift in the spectrum of the haem group in the presence of ATP. Secondly, it is clear that $d\bar{c}_A/d[ATP] < 0$, in agreement with the observation that the weight-average elution volume of the acceptor is greater in the absence of ATP. A direct interpretation of both results is that ATP binds preferentially to the tetrameric form of methaemoglobin.

The result presented in Figure (III-6) serves two purposes. It provides, firstly, an example of the way in which differential chromatography may be used to investigate the effect of a ligand on the extent of association of a polymerising acceptor system. Together with the results obtained with β-lactoglobulin A, it supports the postulate that differential chromatography may find general use as a tool in the study of factors which affect the self-interactions of proteins. Secondly, the finding that ATP binds preferentially to the tetrameric form of

methaemoglobin provides information on the physiologically important interaction between mammalian haemoglobin and organic phosphates, such as ATP and 2,3-diphosphoglyceric acid. Further studies of this interaction, using purified haemoglobin A rather than the pooled haemoglobins of a single donor are reported in the next Chapter.

CHAPTER IV

THE INFLUENCE OF ORGANIC PHOSPHATE EFFECTORS ON THE DISSOCIATION OF ADULT HUMAN HAEMOGLOBIN

1. Introduction.

Normal adult haemoglobin occurs in aqueous solution in the erythrocyte at concentrations as high as 32%. Crude haemolysate may be fractionated (Chapter V) into haemoglobin A consisting of two α and two β polypeptide chains, $(\alpha\beta)_2$, and a number of minor variants such as foetal haemoglobin ($\alpha_2\gamma_2$), and haemoglobin $A_2(\alpha_2\delta_2)$ (Schmidt, 1972). The oxygenated form of haemoglobin A is considered to exist in solution at pH 7 predominantly as the $(\alpha\beta)_2$ form of molecular weight 64,500, lower molecular weight forms occurring in appreciable relative amounts only at high dilution (Schachman and Edelstein, 1966; Chiancone *et al.*, 1968) or in high salt concentrations (Rossi-Fanelli *et al.*, 1964; Benesch, Benesch and Macduff, 1964; Guidotti, 1967). Guidotti reports a dissociation constant for the reaction $(\alpha\beta)_2 \rightleftharpoons 2\alpha\beta$ of 1.2×10^{-4} moles/l in 2 M sodium chloride. The state of association of the deoxygenated form at pH 7 is controversial, but Kellett and Gutfreund (1970) and Norén, Ho and Casassa (1971) suggest that dissociation is slight (equilibrium constant $\leq 10^{-6}$ mole/l) even in high salt concentrations, a finding in basic agreement with the osmotic pressure studies of Guidotti (1967). In view of these findings, it appears that the dissociation reaction $(\alpha\beta)_2 \rightleftharpoons 2\alpha\beta$ is not important in relation to the binding of oxygen to deoxyhaemoglobin although it may be a secondary effect following oxygenation.

Sigmoidal oxygen binding curves have frequently been observed with purified haemoglobin in the concentration range 10^{-7} - 10^{-4} M haem (Kellett and Gutfreund, 1970) and numerous investigations including X-ray diffraction (Perutz, 1969,

1970a and b), spin labelling (Ogawa, McConnell and Horwitz, 1968; Baldassare, Charache, Jones and Ho, 1970) and nuclear magnetic resonance (Ho *et al.*, 1970; Ogawa and Schullman, 1971) have attempted to explain the phenomenon. From a detailed analysis of X-ray crystallographic results obtained with oxy- and deoxyhaemoglobin, Perutz (1970a) suggests that the binding of oxygen causes a shift of the iron atom relative to the plane of the porphyrin ring. This shift is accompanied by a reversible transition in the tertiary structure of the α and β subunits and in their quaternary arrangement within the tetramer. As Arnone (1972) points out and Perutz (1970b) implies, the acceptor system may be visualised, in terms of the model of Monod *et al.* (1965), as a mixture of two isomeric states with pronouncedly different affinities for the ligand oxygen. More detailed mechanisms invoking haem-haem interactions (cooperative binding sites) have also been proposed (Koshland *et al.*, 1966).

While these elegant descriptions account for oxygen binding results obtained with purified haemoglobin A, they do not completely explain the markedly sigmoidal binding curve observed with intact erythrocytes. With the latter, the point of inflection of the binding curve is shifted to much higher oxygen pressures so that oxygen is released at physiological tensions which differ little from those encountered in the lung tissue (Brewer and Eaton, 1971). If haemoglobin behaves according to the model of Monod *et al.* (1965), any factor which affects the magnitude of the constant governing the relative amounts of the two

conformational states of the tetramer will also affect the form of the binding curve (Chapter I). Among the possible variables such as ionic strength or pH (the Bohr effect) the concentration of organic phosphates is of particular significance. Since the early discovery that 2,3-diphosphoglyceric acid (DPG) occurs in high concentration in the erythrocytes of several species (Greenwald, 1925; Rapoport and Guest, 1941), several recent studies (Benesch and Benesch, 1967; Benesch, Benesch and Yu, 1968; Benesch, Benesch, Renthal and Gratzer, 1971) have shown that the "right-shift" in the oxygen binding curve in the erythrocyte can be simulated with purified haemoglobin by including increasing concentrations of the organic phosphate in the reaction mixture. It is now known (Brewer and Eaton, 1971) that the red blood cells of several mammalian species possess the enzymatic machinery necessary for the production of organic phosphates, notably DPG and adenosine 5'-triphosphate (ATP), the latter also functioning as an inhibitor of oxygen binding (Chanutin and Curnish, 1967; Benesch and Benesch, 1967). Recent X-ray diffraction data on the complex between DPG and human deoxyhaemoglobin shows that one molecule of DPG occupies a stereochemically complementary position in the central cavity on the two-fold symmetry axis of the tetramer (Arnone, 1972). The DPG appears to be held by electrostatic interactions with several cationic groups on the β chains. These include the valines 1, histidines 2 and 143 and one of the lysines 82 (Figure 3; Arnone, 1972). Similar detail is not available on the binding of ATP to the tetramer. Perutz (1970b) and Arnone (1972) have noted that the complementary stereochemistry is specific for the

deoxystructure, and is lost on oxygenation. In terms of the model of Monod *et al.* (1965), Arnone visualises the effector stabilising the deoxy structure (equation I-54 with n = 1). Thus haemoglobin is considered to exist in solution as an equilibrium mixture of two conformational states A and C, the A state binding oxygen (S) preferentially, the C state preferring the effectors, (E), ATP or DPG.

In the present study, experiments were performed on aqueous solutions of methaemoglobin in which the ferrous ion is oxidised to the ferric state. In the absence of a reducing agent methaemoglobin is formed spontaneously from oxyhaemoglobin, and this reaction was taken to completion with potassium ferricyanide to give a chemically well-defined product. Perutz (1970a) notes that the quaternary structure of the haemoglobin tetramer is dictated primarily by the state of coordination of the iron atoms and not by their valency state. Since methaemoglobin has a water molecule liganded to each ferric iron in lieu of an oxygen molecule, he concluded that methaemoglobin had the same quaternary structure as oxyhaemoglobin, which would imply that DPG or ATP would not bind strongly between the two β chains of the met derivative at pH 7. Very recently, however, Perutz (1972) has modified his earlier claim and demonstrated a reciprocal relationship between the quaternary structure and the spin state of the iron atoms. In methaemoglobin, liganded with water, the iron atoms occupy an intermediate position between their out-of-plane position in the deoxy form (high spin) and their planar position in oxyhaemoglobin (low spin). If the reciprocal relationship suggested by

Perutz is correct, it is reasonable to assume that methaemoglobin in aqueous solution exists either as an equilibrium mixture of the A and C states discussed above, or as a flexible conformational state which, on binding DPG or ATP, would adopt the quaternary structure (and hence spin state) of deoxyhaemoglobin (C). One basic question which must be answered before the detailed quaternary structure of methaemoglobin *in aqueous solution* and other problems are clarified, is whether or not ATP and DPG can cross-link the β chains of aquo-methaemoglobin. Chanutin and Hermann (1969) have shown that these organic modifiers do, in fact, bind to methaemoglobin, but have not established that they cross-link the β chains. While the dissociation reaction $(\alpha\beta)_2 \rightleftharpoons 2\alpha\beta$ is probably of little biological significance, it does provide an excellent probe for investigating, in solution, the factors which affect the interactions between the amino residues involved in the tetramer formation. These interactions, of course, involve residues on both α and β chains and control experiments with the isolated chains are required to define their roles in the association. Since the dissociation constant of haemoglobin is extremely small at pH 7, experiments have been conducted in the presence of 0.25 M sodium acetate at pH 5.4, where the relative proportion of dimer, at least of oxy- and carbonmonoxy-haemoglobin, is considerably increased (Field and O'Brien, 1955; Chiancone and Gilbert, 1965).

2. Experimental results.

(a) Characterisation of the dissociation of methaemoglobin at pH 5.4

(i) Frontal gel chromatography. Since relatively large volumes of protein solution are required to maintain a plateau of original concentration in frontal analysis experiments, pooled methaemoglobin, rather than the purified A variant, was investigated by this method. Weight-average elution volumes were obtained from the advancing side of the elution profiles by the use of equation (II-23) and plotted as a function of the corresponding plateau concentration (Figure IV-1). V_w is a sharply decreasing, then slightly increasing function of concentration, a result conforming to the behaviour expected for an associating-dissociating system (*cf.* Figures 1 and 2 of Chiancone *et al.*, 1968). Attempts were made to fit the results with a single dimerisation constant $X = [C]/[A]^2$, where C corresponds to $(\alpha\beta)_2$. A value of 20.4 ml for V_A at infinite dilution was found by extrapolation, and V_A at finite concentrations was calculated according to the relation $V_A = 20.4\ (1 + 0.01\ c)$ where the value of the concentration dependence term, 0.01 dl/g, was taken from the work of Chiancone *et al.* (1968) on oxyhaemoglobin. The corresponding expression for the tetramer, $V_C = 17.4$ $(1 + 0.01\ c)$ was derived from the elution profiles using equation (II-25) and assuming the same concentration dependence. The solid lines in Figure (IV-1) were computed using these relationships and values of X of 3.5×10^3 l/mole and 4.2×10^3 l/mole. In either case there is

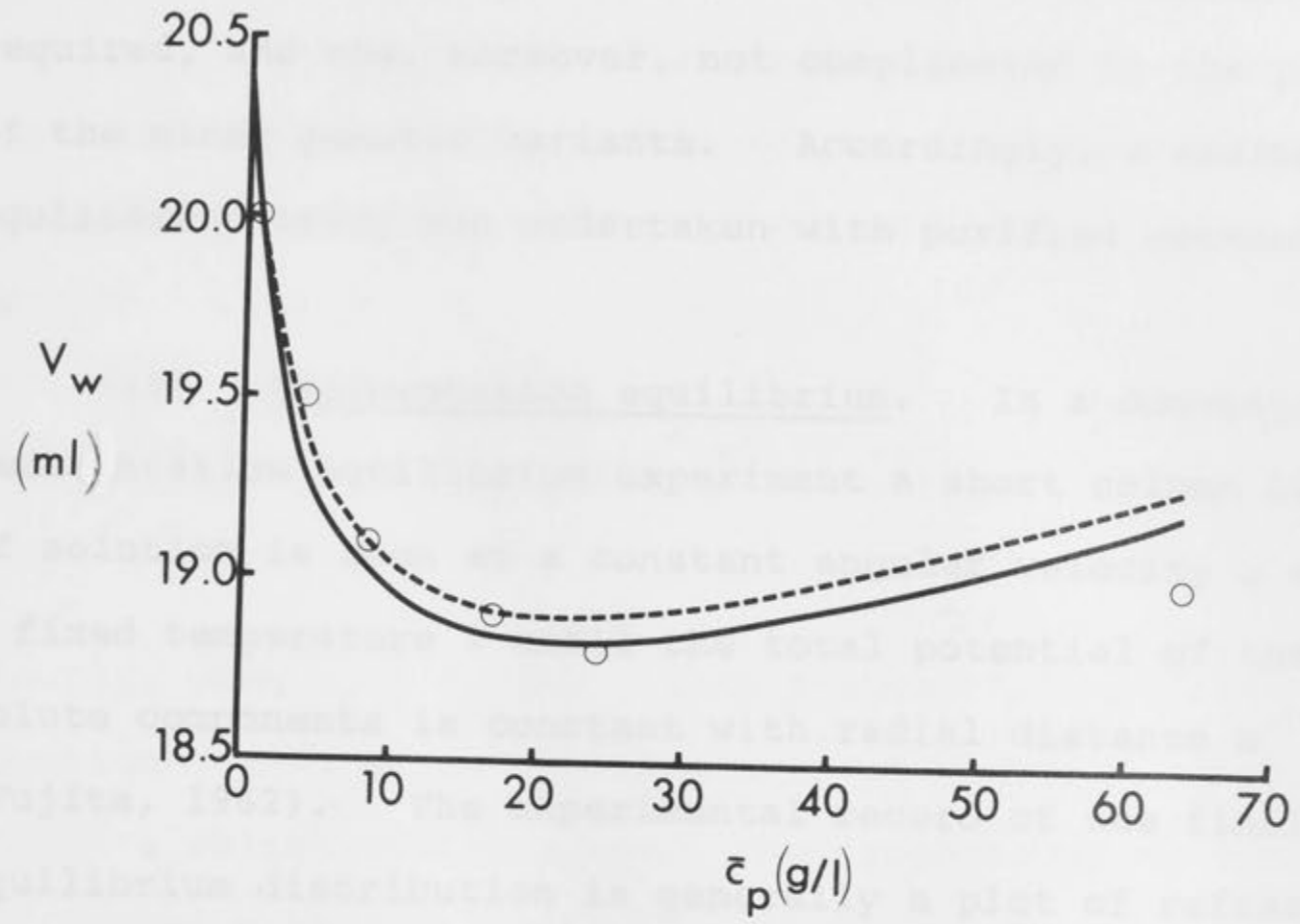


FIGURE IV-1

The dependence of the weight-average elution volume on the total (plateau) concentration, $\bar{c}_p$, of methaemoglobin in 0.25 M sodium acetate buffer, pH 5.4. All measurements were made at 20° with a 27.9 cm x 1.3 cm column of Sephadex G-75. Theoretical curves were constructed using $V_A = 20.4\ (1 + 0.001\bar{c}_p)$ mls, $V_C = 17.4\ (1 + 0.001\bar{c}_p)$ mls and $X = 4.2 \times 10^3$ (——) or 3.5×10^3 (---) l/mole.

reasonable agreement with the experimental points, particularly at concentrations below 30 g/l. However, for the calculations which follow, a more reliable estimate was required, and one, moreover, not complicated by the presence of the minor genetic variants. Accordingly, a sedimentation equilibrium study was undertaken with purified methaemoglobin A.

(ii) Sedimentation equilibrium. In a conventional sedimentation equilibrium experiment a short column length of solution is spun at a constant angular velocity ω and a fixed temperature T until the total potential of the solute components is constant with radial distance x (Fujita, 1962). The experimental record of the final equilibrium distribution is generally a plot of refractive index, in Rayleigh interference fringes, as a function of x, and may be transformed into a plot of total weight concentration $\bar{c}$ *vs.* x by assuming each macromolecular solute has the same specific refractive increment (Chapter V). For a single ideal solute (activity coefficient unity) the equation describing this distribution is well known (Svedberg and Pedersen, 1940; Fujita, 1962).

$$c_i(x_1) = c_i(x_2)e^{\phi_i M_i (x_1^2 - x_2^2)} \qquad \text{(IV-1a)}$$

where x_1 and x_2 are any two radial distances between or at the meniscus, x_m, and the base of the solution, x_b, and

$$\phi_i = \omega^2(1-\bar{v}_i\rho)/2RT \qquad \text{(IV-1b)}$$

ρ being the density of the solution, and $\bar{v}_i$ the partial specific volume of species i. Adams and Fujita (1963) and Nichol and Ogston (1965c) have shown that equation (IV-1) describes the equilibrium distributions of A and C even when they interact ($nA \rightleftharpoons C$). This may be shown simply by writing equation (IV-1a) with i = A and with i = C and forming the ratio

$$\frac{c_C(x_1)}{c_A^n(x_1)} = \frac{c_C(x_2)}{c_A^n(x_2)} e^{M_C(x_1^2-x_2^2)(\phi_C-\phi_A)} \qquad \text{(IV-2)}$$

Clearly, when $\phi_A = \phi_C$ (no volume change on reaction) equation (IV-2) shows that a single equilibrium constant X' (on a weight-scale) governs the proportions of A and C at all points within the cell in accordance with the law of mass action. When $\phi_A \neq \phi_C$ the equilibrium constant becomes a function of radial distance x, a situation still described by equations (IV-1) and (IV-2) and discussed in more detail by Howlett *et al.* (1970).

Equation (IV-1a) may also be written in differential form and summed over all species to yield

$$\sum_i \frac{dc_i(x)}{d(r^2)} = \sum_i \phi_i M_i c_i(x) \qquad \text{(IV-3)}$$

which may be written, when all ϕ_i are equal, as

$$\frac{d\ln\bar{c}}{d(r^2)} = \phi_A M_w(\bar{c}) \qquad \text{(IV-4)}$$

where $M_w(\bar{c})$ is the weight-average molecular weight corresponding to a total concentration $\bar{c}$.

Figure (IV-2a) presents a plot of $\bar{c}(x)$ *vs.* x obtained from a sedimentation equilibrium experiment conducted with methaemoglobin A (initial concentration 4.4 g/l, 9,000 r.p.m., 20°). A detailed account of the method used to obtain this result from the Rayleigh interferogram is given in Chapter V. The results were replotted in the form $\ln\bar{c}$ *vs.* x^2 and equation (IV-4) together with a value of 0.749 for $\bar{v}$ (Svedberg and Pedersen, 1940) was used to calculate values of the apparent M_w as a function of x and hence of $\bar{c}(x)$. From the plot of $(M_w)_{app}$ *vs.* $\bar{c}(x)$ in Figure (IV-2b) it is clear that dilution favours dissociation into the A (= $\alpha\beta$) form. The equilibrium constant was obtained from the results shown in Figure (IV-2a) by a modification of the procedure described by Chun and Kim (1970). The total concentration at any position x, may be written directly as

$$\bar{c}(x_1) = c_A(x_1) + c_C(x_1) \qquad \text{(IV-5)}$$

and by means of equation (IV-1a) the total concentration at a second radial position x_2 may be written

$$\bar{c}(x_2) = c_A(x_1)e^{\phi_A M_A(x_2^2-x_1^2)} + c_C(x_1)e^{\phi_A M_C(x_2^2-x_1^2)} \qquad \text{(IV-6)}$$

In the present situation A = $\alpha\beta$, C = $(\alpha\beta)_2$ and hence M_A, M_C are known and the values of the exponentials in equation (IV-6) can be calculated. Equations (IV-5) and (IV-6) can then be solved simultaneously for $c_A(x_1)$ and $c_C(x_1)$ and hence X' at x_1. Chun and Kim (1970) suggested that this procedure be repeated at various values of x in order to obtain a set of equilibrium constants down the cell. This worker has

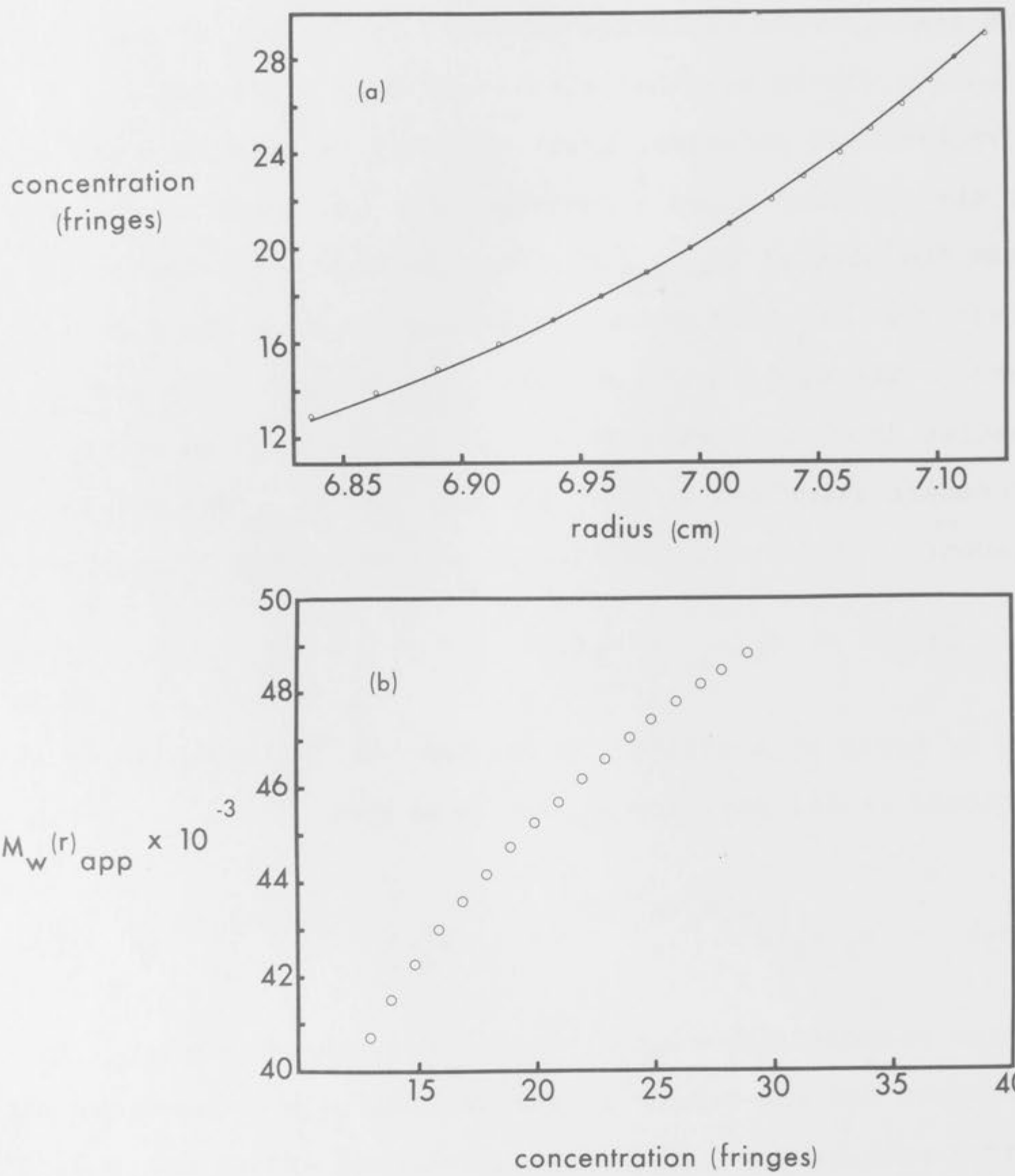

concentration
(fringes)
28
24
20
16
12
(a)
6.85
6.90
6.95
7.00
7.05
7.10
radius (cm)
$M_w(r)_{app} \times 10^{-3}$
50
48
46
44
42
40
(b)
15
20
25
30
35
40
concentration (fringes)

FIGURE IV-2

Sedimentation equilibrium results obtained with methaemoglobin A in 0.25 sodium acetate, pH 5.4, at 20°.

(a) The equilibrium concentration distribution (o) obtained when methaemoglobin (initial concentration 4.4 g/l) was examined at an angular velocity of 9,000 r.p.m. The solid curve was computed with a value of 0.258 l/g (4.15×10^3 l/mole) for the equilibrium constant describing the dimer-tetramer association reaction.

(b) The apparent weight-average molecular weight of methaemoglobin plotted as a function of total protein concentration.

proposed a scheme for estimating $c_A(x_1)$ and $c_C(x_1)$, in which all the experimental data is used simultaneously. Equation (IV-5), and equation (IV-6) written for every other experimentally determined point $(x,\bar{c})$ may be represented in matrix notation by

$$\underset{\sim}{G\beta} = \underset{\sim}{c}$$

$$G = \begin{pmatrix} 1 & 1 \\ e^{\phi_A M_A (x_2^2 - x_1^2)} & e^{\phi_A M_C (x_2^2 - x_1^2)} \\ e^{\phi_A M_A (x_3^2 - x_1^2)} & e^{\phi_A M_C (x_3^2 - x_1^2)} \\ \vdots & \vdots \end{pmatrix} \qquad \text{(IV-7)}$$

$$\underset{\sim}{c} = \begin{pmatrix} \bar{c}(x_1) \\ \bar{c}(x_2) \\ \vdots \end{pmatrix} \quad \text{and} \quad \underset{\sim}{\beta} = \begin{pmatrix} c_A(x_1) \\ c_C(x_1) \end{pmatrix}$$

where all elements of G and $\underset{\sim}{c}$ are known and $\underset{\sim}{\beta}$ is to be determined. The problem is immediately recognisable as one of multiple linear regression, and the least squares estimate $\underset{\sim}{\hat{\beta}}$ of $\underset{\sim}{\beta}$ is given by,

$$\underset{\sim}{\hat{\beta}} = (G^T G)^{-1} G^T \underset{\sim}{c} \qquad \text{(IV-8)}$$

where superscripts T and -1 represent the matrix operations of transposition and inversion respectively. The estimates of $c_A(x_1)$ and $c_C(x_2)$ may then be used to calculate an X' which is applicable at all radial levels x_i within the cell. The point $(x_1,\bar{c}(x_1))$ may be chosen at will from the experimental data without affecting the estimate of X',

TABLE IV-1

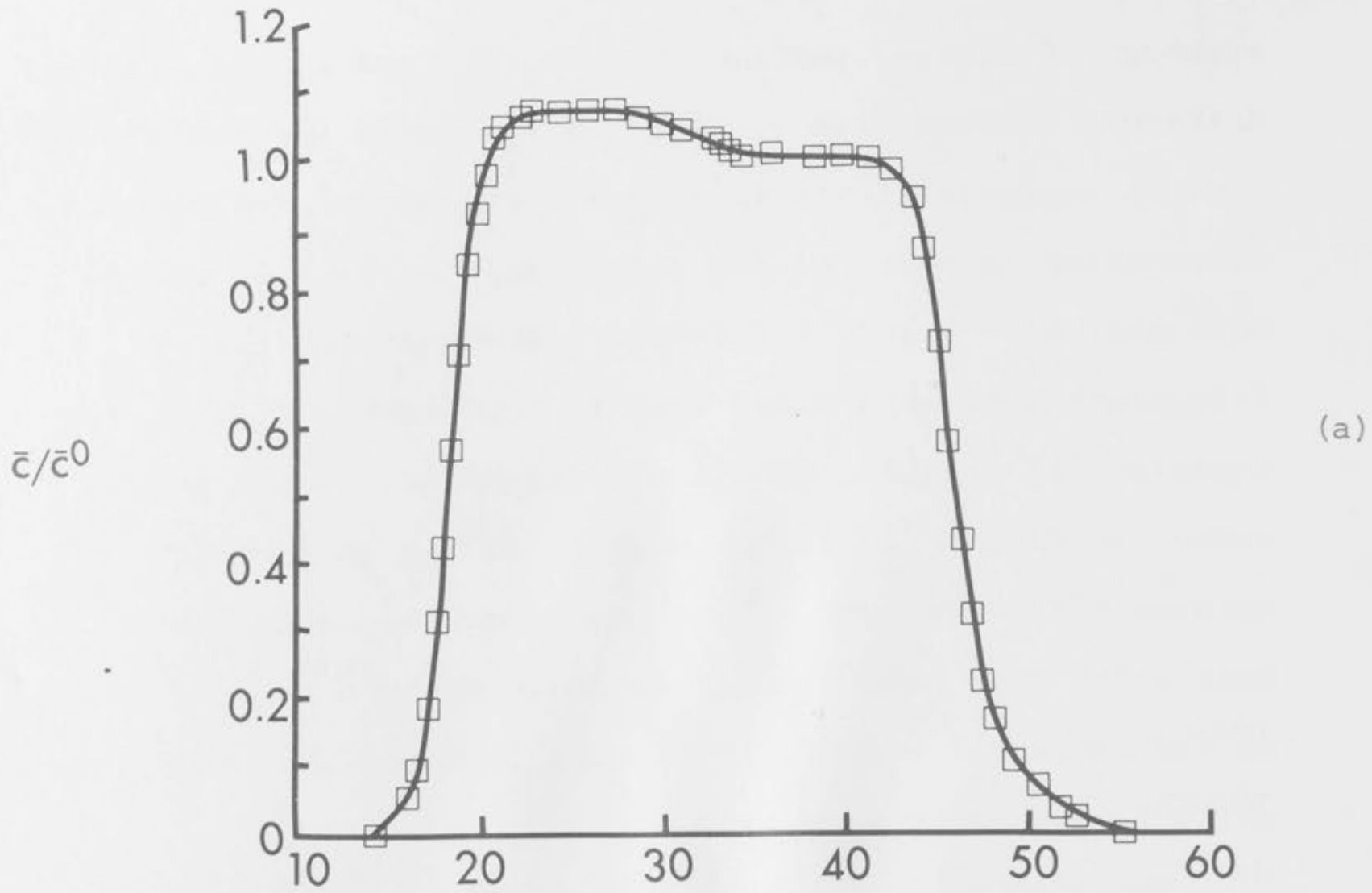
The analysis of the sedimentation equilibrium data presented in Figure (IV-2a) according to the modified procedure of Chun and Kim (1970).

r(cm)	$\bar{c}$	c_A (fringes)	c_C	X'(l/g)
6.837	12.95	8.62	4.25	0.25761
6.864	13.95	9.10	4.74	0.25759
6.891	14.95	9.61	5.29	0.25759
6.916	15.95	10.11	5.85	0.25760
6.939	16.95	10.57	6.40	0.25759
6.959	17.95	11.03	6.96	0.25759
6.978	18.95	11.46	7.51	0.25761
6.997	19.95	11.89	8.09	0.25758
7.013	20.95	12.30	8.66	0.25761
7.031	21.95	12.75	9.31	0.25758
7.046	22.95	13.14	9.89	0.25760
7.060	23.95	13.55	10.50	0.25758
7.072	24.95	13.89	11.04	0.25759
7.087	25.95	14.30	11.70	0.25760
7.098	26.95	14.65	12.28	0.25758
7.109	27.95	14.97	12.82	0.25760
7.122	28.95	15.37	13.52	0.25761

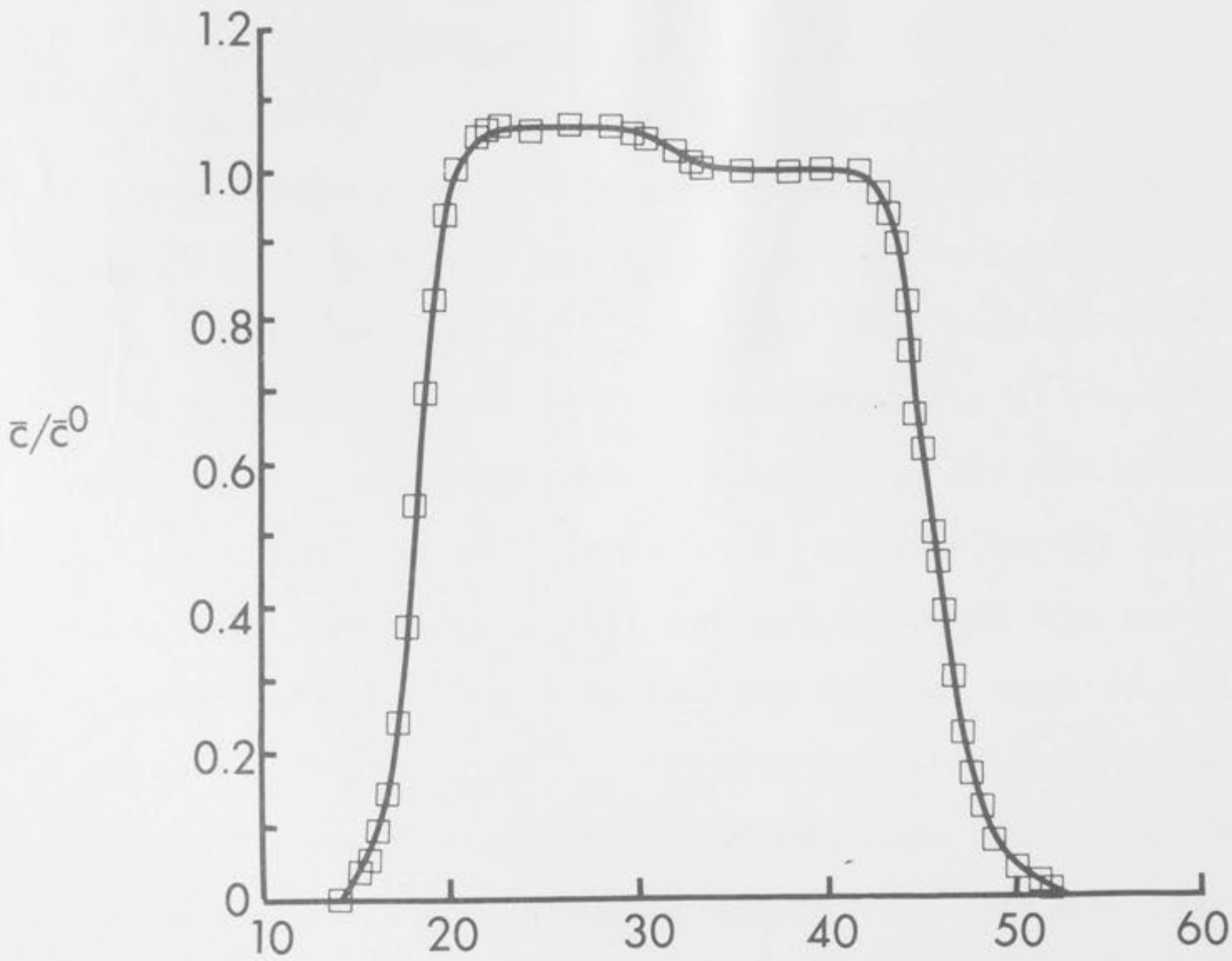
although of course, the estimates $c_A(x_1)$ and $c_C(x_1)$ will be different in each case. Table (IV-1) shows the results of such an analysis of the data in Figure (IV-2a) taking each observation in turn, as the point $(x_1, \bar{c}(x_1))$. It can be seen that X' = 0.2576 ± 0.0002 l/g throughout. To illustrate that this value does in fact describe all experimental results, it was used to construct the solid curve in Figure (IV-2a) from which it can be seen the agreement is excellent. The corresponding association constant X on a molar scale was found to be 4.15×10^3 l/mole, in the range $3.5 - 4.2 \times 10^3$ l/mole indicated by the frontal gel chromatography results.

(b) <u>The effect of adenosine 5'-triphosphate and 2,3-diphospho-D-glycerate on the dissociation of methaemoglobin A at pH 5.4 in acetate buffer</u>

(i) <u>Differential chromatography</u>. In two separate experiments, columns of Sephadex G-75 were equilibrated with acetate buffer pH 5.4, I = 0.25 and approximately 32 ml of reaction mixture were applied. The reaction mixtures contained ~ 1 g/l methaemoglobin in the same buffer and in one case ATP (50 moles/mole of tetramer) and in the other, 2,3-DPG (20 moles/mole of tetramer). The resulting elution profiles are shown in Figures (IV-3, a and b), from which it can be seen that in both cases a protein concentration gradient separates two plateaux. In agreement with the study performed with pooled methaemoglobin and ATP (Figure III-6), the sign of $d\bar{c}/d[E]$ indicates that the extent of association is greater in the regions containing the effectors. Independent spectrophotometric measurements


1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60
$\bar{c}/\bar{c}^0$

(a)


1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60
$\bar{c}/\bar{c}^0$
Elution volume (ml)

(b)

FIGURE IV-3

Elution profiles obtained in the differential chromatography of mixtures of methaemoglobin A and organic phosphate effectors at 20°.

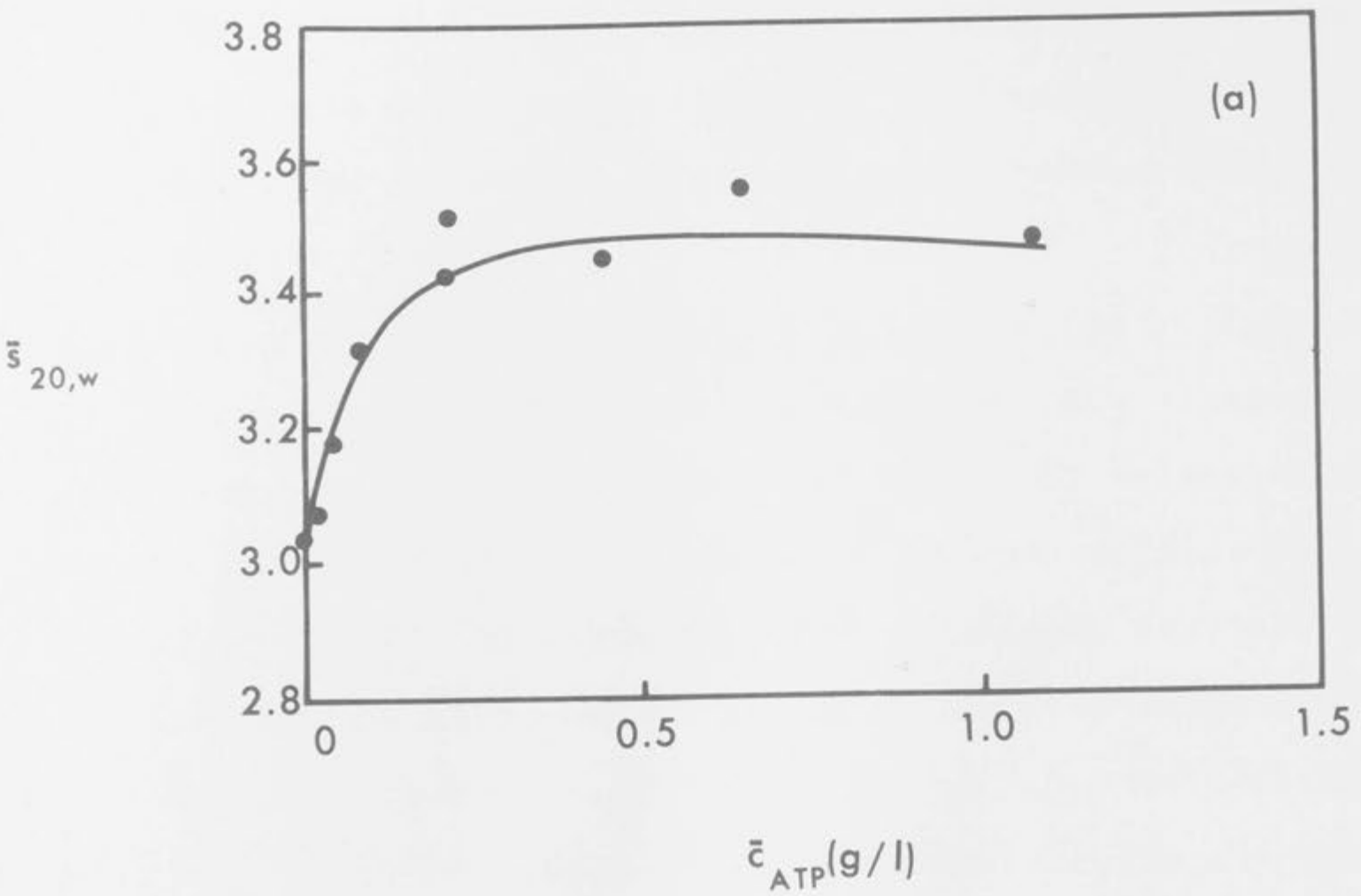
(a) The elution profile resulting from the application of 30.2 ml of a solution of methaemoglobin A (1 g/l) and 0.54 g/l ATP (50 moles/mole of tetrameric haemoglobin) in 0.25M sodium acetate, pH 5.4, to a 28.0 cm x 1.3 cm column of Sephadex G-75 equilibrated with the acetate buffer.

(b) The elution profile obtained in a similar experiment on the same column but with the ATP replaced by 0.073 g/l of DPG (20 moles/mole of tetramer). The loading volume was 28.8 ml.

showed that these results could not be accounted for by a change in extinction associated with effector binding. The basic finding that the addition of these effectors favoured tetramer formation was confirmed by two other methods.

(ii) Sedimentation velocity studies. A series of experiments was conducted in which the methaemoglobin A concentration in the pH 5.4 buffer was held constant (~ 4 g/l) and increasing amounts of either ATP or DPG were included in the reaction mixture. Weight-average sedimentation coefficients were measured and corrected to 20° in water according to equation (II-30). These corrections were small, since the experiments were all conducted at 20°. The results are plotted in Figure (IV-4) as a function of total effector concentration, and it is clear that with both effectors, $\bar{s}_{20,w}$ increases initially with the effector concentration, and, within experimental error attains the same limiting value at high concentration. In section 3(a) of this Chapter these results will be subjected to a quantitative analysis, but for the present it suffices to note that they confirm qualitatively the conclusion drawn from the results presented in Figures (IV-3, a and b).

(iii) Sedimentation equilibrium. In these studies attention was restricted to the effect of ATP on the association of methaemoglobin A, since each experiment must be preceded by dialysis (Casassa and Eisenberg, 1964) and there was insufficient DPG available for the purpose. The dialysate, containing an equilibrium concentration of ATP was used to fill the reference channel, and hence the plots


3.8
3.6
3.4
3.2
3.0
2.8
0
0.5
1.0
1.5
(a)
$\bar{s}_{20,w}$
$\bar{c}_{ATP}$(g/l)

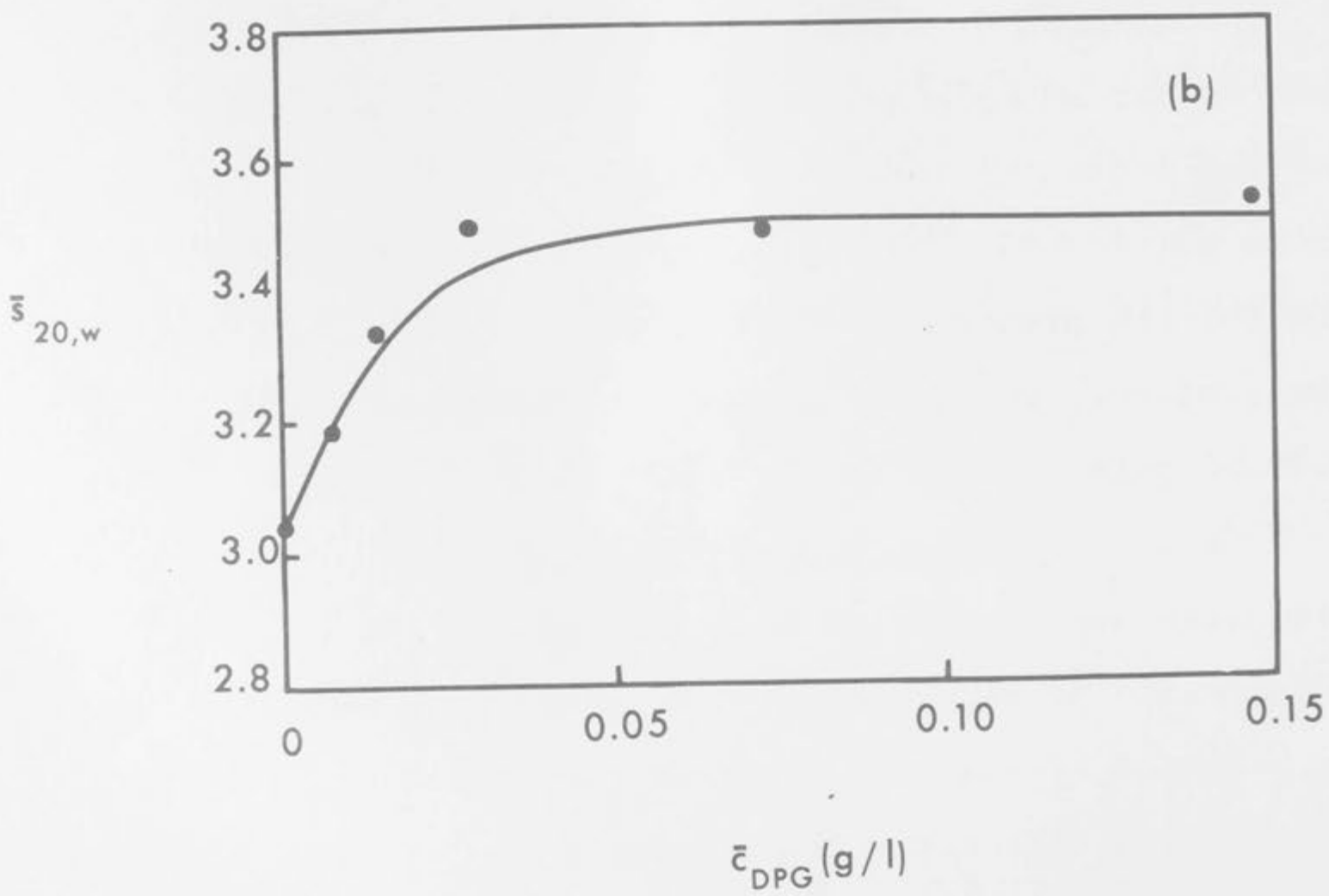

3.8
3.6
3.4
3.2
3.0
2.8
0
0.05
0.10
0.15
(b)
$\bar{s}_{20,w}$
$\bar{c}_{DPG}$(g/l)

FIGURE IV-4

Sedimentation velocity results obtained with methaemoglobin A (4 g/l) in 0.25 M sodium acetate, pH 5.4, in the presence of organic phosphate effectors.

(a) The dependence of the weight-average sedimentation coefficient on the total concentration of ATP. The solid line was computed for the model $2A \rightleftharpoons C$ with ATP binding to one site on A ($N_A = 1.2 \times 10^3$ l/mole) and one site on C ($N_C = 2.1 \times 10^3$ l/mole). A value of 4.15×10^3 l/mole was used for the dimerisation constant X, and s_A and s_C were taken to be 2.40 S and 4.10 S respectively.

(b) The dependence of the weight-average sedimentation coefficient of methaemoglobin A on the total concentration of DPG. The solid line was computed for the same model described in (a) but with values for N_A and N_C of 3×10^3 l/mole and 6×10^4 l/mole respectively.

of $\bar{c}$ *vs.* x determined from the experimental Rayleigh interferograms recorded the constituent concentration of all forms of the acceptor, methaemoglobin A. In Figure (IV-5) weight-average molecular weights are plotted as a function of total concentration (in fringes). The lower plot was obtained in the absence of added effector (Figure IV-2b) and is included for comparison. The upper curves refer to mixtures in which the original concentration of ATP was 5 and 25 moles/mole of tetramer. At first sight it may seem surprising that the latter curves are virtually coincident. A reason for this will be given in section 3(b) which is devoted to a quantitative analysis of the results, but for the present it is simply noted that the addition of ATP effectively increases the apparent weight-average molecular weight.

In conclusion, three different methods, differential chromatography, sedimentation velocity and sedimentation equilibrium all show that the acid dissociation of methaemoglobin A is partially reversed by the addition of the organic phosphates studied. The problem of determining whether this action is mediated via intermolecular linkage of the α chains or the β chains (or both) has been examined using ATP as the effector.

(iv) Sedimentation velocity studies with the isolated α and β chains. Preparations of the individual chains obtained by the methods described in Chapter V were studied at pH 7.0 and pH 5.4, in the presence and absence of ATP. Since this worker encountered considerable difficulty in attempting to convert the isolated chains to the met form,

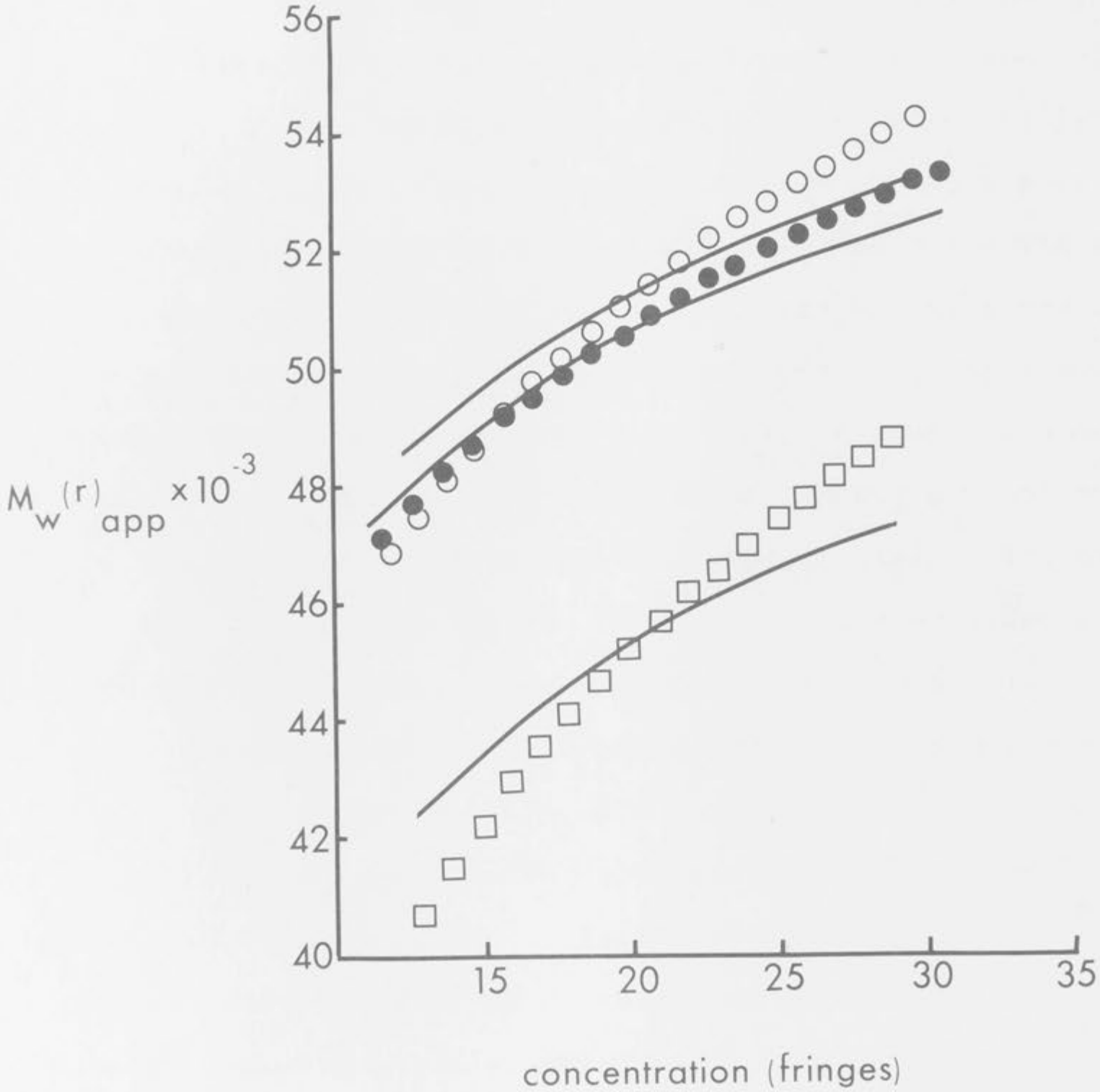

$M_w(r)_{app} \times 10^{-3}$
56
54
52
50
48
46
44
42
40
15
20
25
30
35
concentration (fringes)

FIGURE IV-5.

The effect of ATP on the weight-average molecular weight of methaemoglobin A (initial concentration 4.4 g/l) in 0.25M sodium acetate, pH 5.4, at 20°. Sedimentation equilibrium experiments were performed at 9,000 r.p.m. with solutions containing 5 moles of ATP per mole of tetramer (●) and 25 moles/tetramer (○). The plot (□) obtained in the absence of effector (Figure IV-2b) is included for comparison.

The solid lines were computed for the model 2A C with ATP binding to one site on A (N_A = 1.03 x 10^3 l/mole) and one site on C (N_C = 11.1 x 10^3 l/mole), and employing a value for the dimerisation constant of 4.15 x 10^3 l/mole. The molecular weight of C was taken to be 64,500.

it was decided to work with the stable and soluble carbonmonoxy derivatives which, at least in the tetramer, have a tertiary structure indistinguishable from oxyhaemoglobin (Perutz, 1969). The weight-average sedimentation coefficients observed are reported in Table (IV-2), and several points merit comment.

Firstly, in all experiments involving α chains only single symmetrical boundaries were observed. In the absence of ATP the $\bar{s}_{20,w}$ observed at pH 7.0 agrees with that reported by Briehl (1964), and corresponds to a dimeric form if it is assumed the α chain is monomeric at pH 5.4 ($s_D = s_M\ 2^{2/3}$). This conclusion is, of course, tentative, but the significance of these results is that the addition of ATP does not alter the state of association of the α chains at either pH. The β chains, at pH 7.0, appear to exist as the tetramer (haemoglobin H) both in the presence and absence of ATP, the value of 4.75 being in excellent agreement with that reported by Bucci *et al.* (1965). In the absence of ATP, decreasing the pH results in dissociation, but in contrast to the results obtained with the α chains, it was found that the addition of ATP effected an increase in $\bar{s}_{20,w}$ which was well outside experimental error. It is not claimed that these results can be analysed in terms of the nature and relative proportions of the various association states of β chains existing in solution, but it appears that at pH 5.4 ATP crosslinks β chains alone.

It is, therefore, tentatively concluded that the increase in the extent of association of methaemoglobin A at pH 5.4 (Figures IV-3, IV-4 and IV-5) is mediated by an interaction of ATP with β chains only. With this information, it is

TABLE IV-2

Weight-average sedimentation coefficients of the α and β chains of carbonmonoxyhaemoglobin A in the presence and absence of ATP.

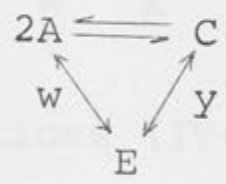
pH	α-chains		β-chains	
	no ATP	2 moles ATP per mole of α	no ATP	2 moles ATP per mole of β
7.0	2.2_9 S	2.2_6 S	4.7_0 S	4.7_5 S
5.4	1.6_0 S	1.5_9 S	2.7_7 S	3.9_2 S

now possible to attempt a quantitative interpretation of the results presented in Figures (IV-4) and (IV-5).

3. A quantitative analysis of sedimentation velocity and equilibrium results obtained with methaemoglobin A at pH 5.4 in the presence of ATP and DPG.

(a) Sedimentation velocity

In this section certain theoretical relationships derived in Chapter I are used, and for convenience, the system under discussion is summarised by



(IV-9)

where w and y are the number of equivalent and independent binding sites per molecule of $A(=\alpha\beta)$ and $C(=(\alpha\beta)_2)$ respectively, and N_A and N_C are the appropriate intrinsic binding constants. In view of the findings of Benesch and Benesch (1969) and of Arnone (1972), it is reasonable to assume that y = 1 and that w equals either one or zero. Accordingly, the present results are examined in terms of two models: Model 1, in which w is taken to be zero and only one parameter, N_C requires estimation; and Model 2 (w = 1) which requires estimates for both N_A and N_C.

(i) Model 1. The appropriate binding equation based on equation (I-11) is

$$r = (\bar{c}_E - M_E[E])/\bar{c}_A = \frac{yM_E N_C [C][E](1+N_C[E])^{y-1}}{M_A[A]+2M_A[C](1+N_C[E])^y} \qquad \text{(IV-10)}$$

where y, the number of binding sites on C, is initially unspecified. In order to express N_C in terms of experimentally available quantities, [A] and [E] are related to the apparent dimerisation equilibrium constant X* by rewriting equations (I-54b) and (I-11) respectively to give

$$[E] = \{(X^*/X)^{1/y}-1\}/N_C \qquad \text{(IV-11)}$$

and,

$$[A] = \{-M_A+(M_A^2+8\bar{c}_A M_A X^*)^{1/2}\}/4M_A X^* \qquad \text{(IV-12)}$$

Combining equations (IV-10), (IV-11) and (IV-12) with the definition $X = [C]/[A]^2$ yields, on extensive rearrangement

$$N_C = \frac{\{(X^*/X)^{1/y}-1\}}{\{\bar{c}_E/M_E - y\bar{c}_A(1-(X/X^*)^{1/y})(\sqrt{\Delta}-M_A)/2M_A(\sqrt{\Delta}+M_A)\}} \qquad \text{(IV-13a)}$$

$$\Delta = M_A^2 + 8M_A X^* \bar{c}_A \qquad \text{(IV-13b)}$$

Since the constants X (= 4150 l/mole) and $\bar{c}_A$ (= 4 g/l) are known, equation (IV-13) may be used to calculate a value for N_C by specifying X* for a given $\bar{c}_E$. Values of $\bar{s}_{20,w}$ are plotted as a function of $\bar{c}_E$ in Figure (IV-4) and application of equation (II-22) written on a molar scale yields X* as a function of $\bar{c}_E$. These values are shown in Table (IV-3) together with N_C calculated for y = 1, 2 and 3. There is evidently considerable scatter in the values of N_C for each of the values of y examined, and, in general, this variation becomes greater as y increases: for example with the effector DPG there are 5-, 9- and 12-fold variations in N_C for y = 1, 2 and 3, respectively.

TABLE IV-3

Analysis of sedimentation velocity results obtained with methaemoglobin A, in terms of a dimerisation $2A \rightleftharpoons C$ (equilibrium constant $X = 4.15 \times 10^3$ l/mole) with organic phosphate effector binding to C alone (y sites, intrinsic binding constant N_C). The initial protein concentration was 4 g/l and the sedimentation coefficients of A and C were taken to be 2.40 S and 4.10 S respectively. X* is the apparent equilibrium constant for the dimerisation reaction in the presence of effector.

(a) DPG

$\bar{s}_{20,w}$	$\bar{c}_{ATP}$ (g/l)	X* x 10^{-4} l/mole	N_C x 10^{-4} l/mole		
			y = 1	y = 2	y = 3
3.19	0.00732	0.654	2.81	1.32	0.868
3.34	0.0146	1.12	4.17	1.81	1.18
3.50	0.0293	2.09	4.40	1.57	0.969
3.49	0.0732	2.01	1.38	0.450	0.265
3.53	0.146	2.38	0.809	0.244	0.140

(b) ATP

$\bar{s}_{20,w}$	$\bar{c}_{ATP}$ (g/l)	X* x 10^{-4} l/mole	N_C x 10^{-3} l/mole		
			y = 1	y = 2	y = 3
3.07	0.0217	0.432	1.43	0.708	0.470
3.18	0.0434	0.632	9.99	4.56	2.95
3.32	0.0868	1.04	14.4	5.84	3.66
3.42	0.217	1.51	9.33	3.31	1.99
3.51	0.217	2.19	15.4	4.90	2.86
3.45	0.434	1.70	5.25	1.76	1.04
3.56	0.651	2.72	6.23	1.78	1.00
3.48	1.08	1.92	2.40	0.765	0.444

(ii) Model 2 (y = w = 1). The fundamental distinction between this and the previous model may be seen in the expression for X*,

$$X^* = X(1+N_C[E])/(1+N_A[E])^2 \qquad \text{(IV-14)}$$

In contrast to model 1 in which X* was a linear function in [E], X* is now a quadratic function in [E]. The expressions for [E] and X* at the turning point in the plot provide a means of determining N_A and N_C. By setting dX*/d[E] (found by differentiating equation IV-14) to zero, it may be shown that

$$[E]_c = (N_C-2N_A)/N_AN_C = (\beta-2)/N_C \qquad \text{(IV-15a)}$$

$$X^*_c = XN_C^2/4N_A(N_C-N_A) = X\beta^2/4(\beta-1) \qquad \text{(IV-15b)}$$

$$\beta = N_C/N_A \qquad \text{(IV-15c)}$$

From equation (IV-15a) it can be seen that a turning point (in fact a maximum) will be observed if β is greater than 2. The actual value of β may be calculated from the observed X^*_c as the larger root of equation (IV-15b). (The smaller root corresponds to a value of β which is always less than 2, and hence to a negative value for $[E]_c$).

Once a value of β has been estimated the problem becomes, as before, one of determining a value for a single intrinsic binding constant. By substituting the expression for [E] from equation (IV-14) into the appropriate binding equation based on equations (I-11) and (I-8) it follows that

$$N_A = \frac{M_E(\beta X - 2X^* - \sqrt{\Delta_E})/2X^*\bar{c}_A}{\{\bar{c}_E/\bar{c}_A - \frac{M_E(\beta X - 2X^* - \sqrt{\Delta_E})(1+\beta X[A])}{M_A(\beta X - \sqrt{\Delta_E}) + 2M_A\psi X[A]}\}}$$

$$\Delta_E = \beta^2X^2 - 4\beta XX^* + 4XX^* \qquad \text{(IV-16)}$$

$$\psi = 2X^*(1-\beta) + \beta^2X^2 - \beta\sqrt{\Delta_E}$$

$$X[A] = \frac{-M_A(\beta X - \sqrt{\Delta_E}) + \{M_A^2(\beta X - \sqrt{\Delta_E})^2 + 16M_AXX^*\bar{c}_A\psi\}^{1/2}}{4M_A\psi}$$

From the results shown in Figure (IV-4) it is clear that $\bar{s}_{20,w}$ and hence X* for both DPG and ATP tend to a limiting value of approximately 3.50 which is well below that of the sedimentation coefficient of $(\alpha\beta)_2$. Unfortunately precipitation problems prevented examination of these systems at higher effector concentrations keeping $\bar{c}_A$ fixed, and the existence of a true maximum (which may be quite shallow) could not be demonstrated definitively. In order to proceed, limiting values of X* (2.19×10^4 for DPG and 1.93×10^4 for ATP) were estimated from Figure (IV-4) and substituted into equation (IV-15b) to calculate β, and hence N_A from equation (IV-16). These values are summarised in the caption of Figure (IV-4) which compares the experimental results with theoretical curves computed with the reported values. It is clear that the fit is entirely reasonable, suggesting that both ATP and DPG bind to one site on the tetrameric form of methaemoglobin, DPG having the greater affinity, in accord with previous interpretations (Arnone, 1972; Perutz, 1970, 1972). At the same time it appears

that the dimeric form of methaemoglobin also binds the effectors, but with affinities approximately 20 fold less than those of the tetramer. The sedimentation equilibrium data will now be examined in terms of this model.

(b) Sedimentation equilibrium

It is possible to analyse the results presented in Figure (IV-5) in terms of the parameters reported for model 2 provided the concentration distribution of each species can be computed. The procedure is as follows. The relevant species coexisting in equilibrium are A, AE, C, CE and E, hereafter denoted by the general symbol i where i = 1 = A, i = 2 = AE, etc. According to Howlett *et al.* (1970), the amount (in grammes) of each of these species in the cell at equilibrium is given by

$$(Q_i)_{cell} = \theta b \int_{x_m}^{x_b} x c_i(x)\,dx \qquad \text{(IV-17)}$$

where θ is the sector angle (radians) of the cell, and b is the cell thickness. Equation (IV-1) also applies to each species, and may be combined with equation (IV-17) to give, on integration

$$(Q_i)_{cell} = c_i(x_b)\theta b\{1-e^{\phi_i M_i (x_m^2-x_b^2)}\}/2\phi_i M_i \qquad \text{(IV-18)}$$

If the value of $c_i(x_b)$ could be determined, equation (IV-1) could be employed to simulate the required distribution. This value is not directly available from equation (IV-18) however, because $(Q_i)_{cell}$, which is not equal to the total

quantity of species i in an identical volume of the initial mixture undisturbed by the centrifugal field, (Adams, 1964), is unknown. Howlett *et al.* (1970) evaluated $(Q_i)_{cell}$ by solving the following simultaneous equations;

$$(Q_p)_{total} = (Q_A)_{cell} + (Q_C)_{cell} + (Q_{AE})_{cell} M_A/M_{AE} + (Q_{CE})_{cell} M_C/M_{CE}$$

$$(Q_E)_{total} = (Q_E)_{cell} + (Q_{AE})_{cell} M_E/M_{AE} + (Q_{CE})_{cell} M_E/M_{CE}$$

$$(Q_C)_{cell}/(Q_A)^2_{cell} = Z_1 \qquad \text{(IV-19)}$$

$$(Q_{AE})_{cell}/(Q_A)_{cell}(Q_E)_{cell} = Z_2$$

$$(Q_{CE})_{cell}/(Q_C)_{cell}(Q_E)_{cell} = Z_3$$

The first two equations of this set express conservation of mass, and the quantities $(Q_p)_{total} = \bar{c}_A V_{cell}$ and $(Q_E)_{total} = \bar{c}_E V_{cell}$ are known experimental constants. The right-hand side of each of the last three equations may also be evaluated from known data. According to equation (IV-18), Z_1, for example, is given by

$$Z_1 = \frac{c_C(x_b)(1-e^{\phi_C M_C (x_m^2 - x_b^2)})(2\phi M_A)^2}{c_A^2(x_b)\theta b(1-e^{\phi_A M_A (x_m^2 - x_b^2)})^2(2\phi_C M_C)} \qquad \text{(IV-20)}$$

in which the only unknowns $c_A(x_b)$ and $c_C(x_b)$ occur in the ratio, $c_C(x_b)/c_A^2(x_b)$ which may be replaced by X'. Z_2 and Z_3 may be similarly evaluated in terms of the selected values of N_A and N_C, expressed on a weight-concentration scale.

In summary, the simulation proceeds by solving equations (IV-19) for the $(Q_i)_{cell}$, (IV-18) for the $c_i(x_b)$, and employing equation (IV-1) to compute the concentration distribution of each species. For comparison with the experimental results in which the contribution of unbound E was subtracted optically, a quantity $\bar{M}_{app}(x)$ is defined as

$$\bar{M}_{app}(x) = \sum_{i \neq E} M_i c_i(x) / \sum_{i \neq E} c_i(x) \qquad \text{(IV-21)}$$

and evaluated at each x, from the computed simulations.

With the aid of a computer, a range of values of β and N_A were searched using the above procedure. The best fit, shown by the solid lines in Figure (IV-5) was obtained with $N_A = 1.03 \times 10^3$ l/mole and $N_C = 11.1 \times 10^3$ l/mole. The curve corresponding to $\bar{c}_E = 0.217$ g/l fits the experimental data (●) with a maximum error of 1.4% while that corresponding to $\bar{c}_E = 1.09$ g/l deviates maximally by approximately 4% from the experimental points (○). Such deviations are not unreasonable for weight-average molecular weight determinations in which second-order effects such as non ideality, possible volume changes and density variations have been neglected.

Two final points merit comment. Firstly, the correlation between the sedimentation velocity and sedimentation equilibrium results obtained with ATP is quite good, both sets of data being described by $y = w = 1$, $N_A = 1.03 - 1.20 \times 10^3$ l/mole and $N_C = 1.1 - 2.1 \times 10^4$ l/mole. Secondly, an explanation has been found for the apparent inability of a 25 molar excess of ATP to increase

the extent of association of methaemoglobin at pH 5.4 over and above that due to a 5-molar excess (Figures IV-4 and IV-5). It is, simply, that while one site has been lost on the formation of tetramer, the latter has a very much higher affinity for the effector than the dimer. In the sedimentation equilibrium studies the effector concentrations chosen were such that X* was closely approaching its limiting value of X_c.

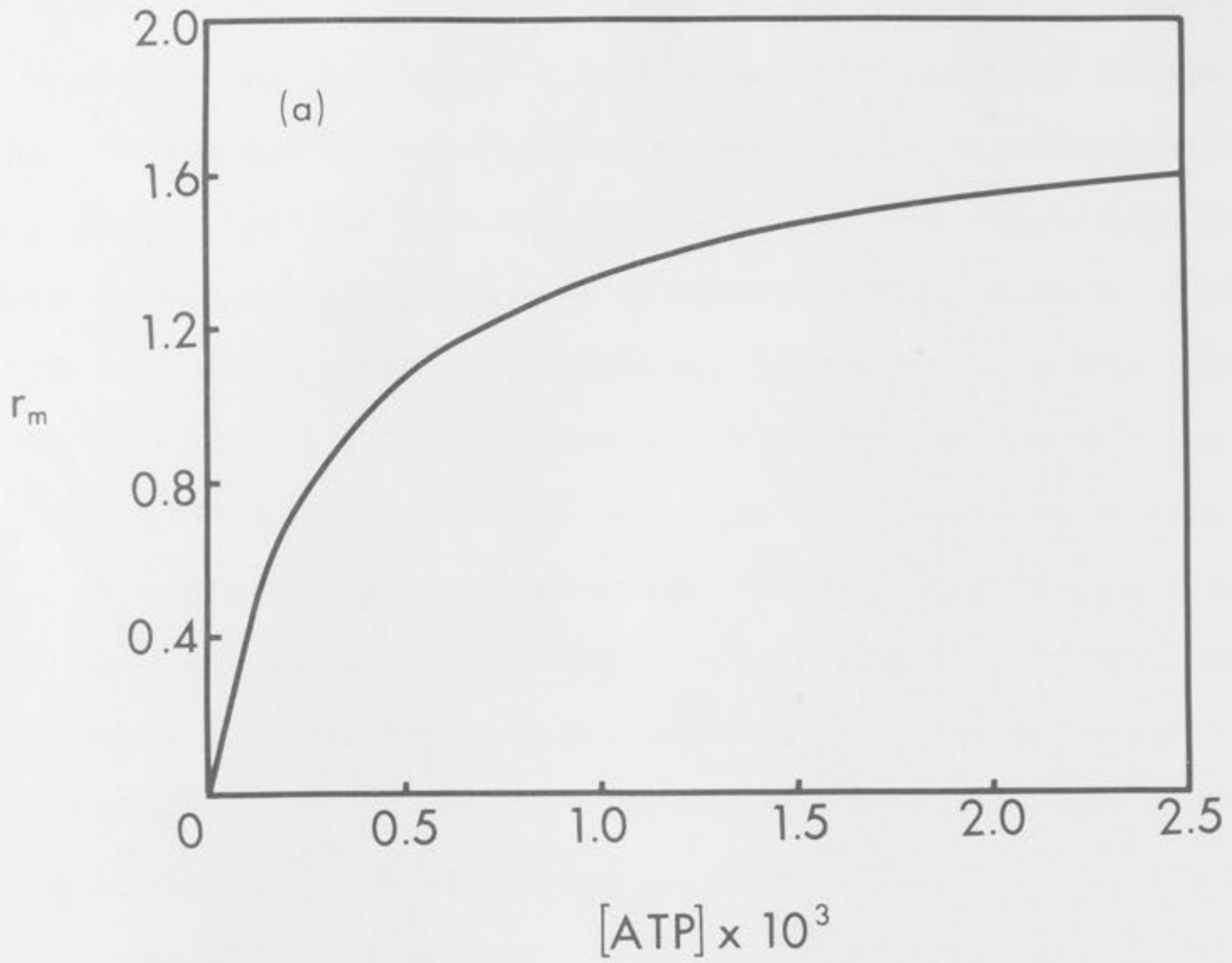
4. General discussion

While the acid dissociation of methaemoglobin is unlikely to be of any biological significance, it has served as a useful probe to show that *in aqueous solution* the organic phosphate effectors, ATP and DPG, favour the formation of the $(\alpha\beta)_2$ tetramer. In agreement with the conclusions of Perutz (1970b) and Arnone (1972), the linkage appears to involve only the β chains, and one binding site on the tetramer. The results also show that at pH 5.4 the αβ units are capable of binding at least one molecule of effector, although with a lower affinity than the tetramer. With this information and the material in Chapter I, it is possible to comment on the nature of the binding curve which would be obtained by equilibrium dialysis experiments with methaemoglobin and ATP or DPG at pH 5.4. Equation (I-39), which defines the turning point in a Scatchard plot, simplifies, with n = 2 and w = y = 1 to

$$(2XN_C[A]+N_A)^2 = 0 \qquad \text{(IV-22)}$$

(While equation (I-39) was originally written in terms of the constants describing the binding of ligand S, it nevertheless

applies to the binding of any ligand, and is written here in terms of effector binding constants, for consistency in presentation). Since the concentration of unbound A cannot be negative it follows from equation (IV-22) that there can be no maximum in the Scatchard plot, and, by inference that there can be no point of inflection in the direct plot of r *vs.* the concentration of unbound effector. Figure (IV-6a) illustrates this point with the binding curve constructed for the haemoglobin - ATP system at pH 5.4, by means of equations (I-11) and (I-8). The total protein concentration was taken to be 4 g/l and the values used for the binding parameters were those found by analysis of the sedimentation equilibrium data: $w = y = 1$, $N_A = 1.03 \times 10^3$ l/mole and $N_C = 11.1 \times 10^3$ l/mole. While the binding curve may closely resemble a rectangular hyperbola, the nonlinearity at high unbound [ATP] concentrations in the corresponding double-reciprocal plot (Figure IV-6b) indicates that, in fact, it deviates from this form. The correct interpretation of such plots, considered alone, is exceedingly difficult. Thus an experimenter may erroneously attribute the deviation to negative cooperativity (Levitzki and Koshland, 1969), or if experimental error obscures the deviation, he may be in danger of interpreting the results in terms of equivalent and independent sites on a single non-interacting acceptor, thereby obtaining incorrect values for the number of binding sites and the intrinsic binding constant. It would appear, therefore, that direct binding studies are inferior to the methods of sedimentation velocity and sedimentation equilibrium in their ability to characterise the interaction parameters for this system.


(a)
2.0
1.6
1.2
0.8
0.4
r_m
0
0.5
1.0
1.5
2.0
2.5
[ATP] x 10^3

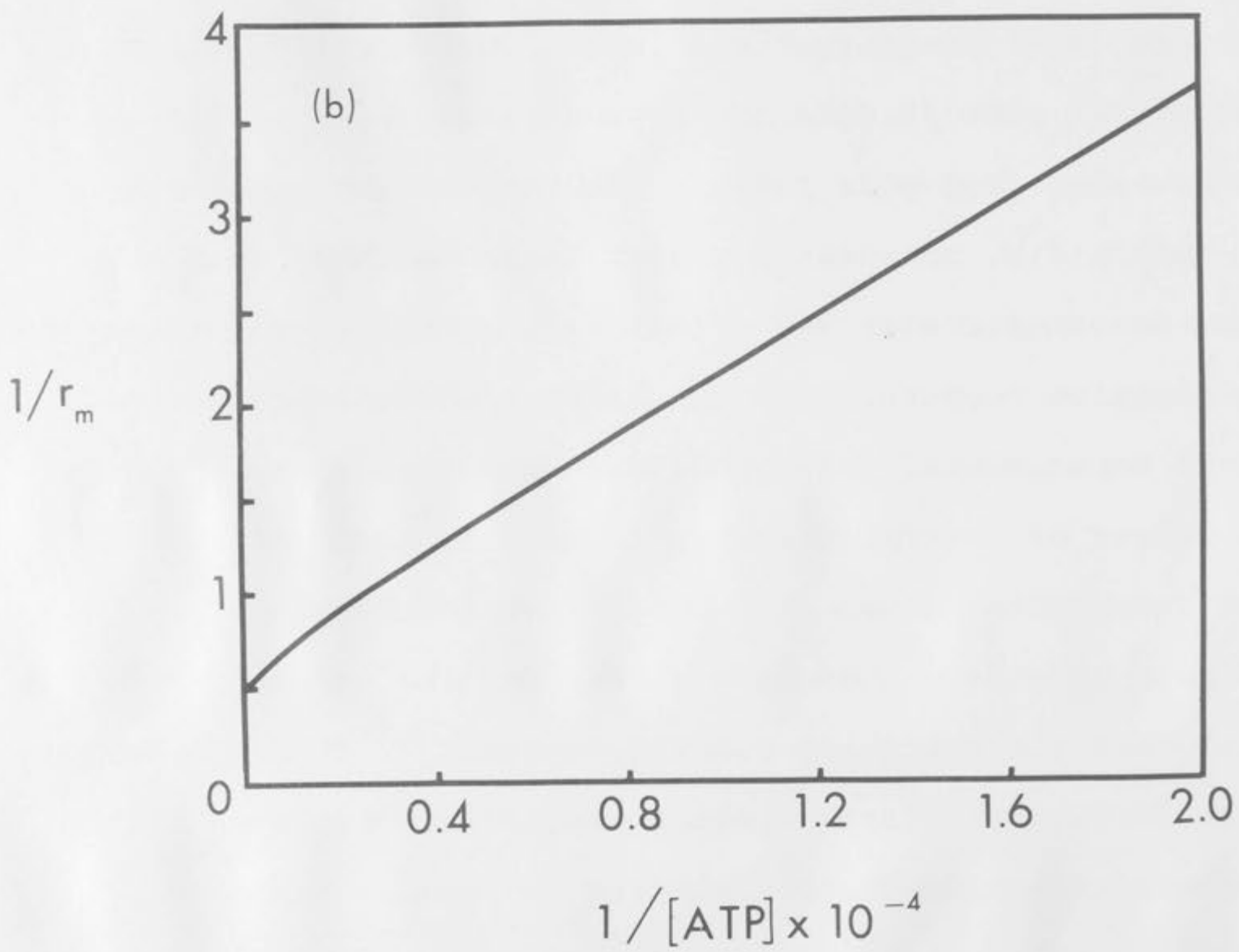

(b)
4
3
2
1
0
$1/r_m$
0.4
0.8
1.2
1.6
2.0
$1/[ATP] \times 10^{-4}$

FIGURE IV-6

(a) A theoretical curve describing the binding of ATP to methaemoglobin A in 0.25M sodium acetate, pH 5.4. The total acceptor concentration was taken to be 4 g/l and the equilibrium constants employed in the simulation were those reported in the caption to Figure IV-5 .

(b) The double-reciprocal plot of the curve in (a).

Certain difficulties arise in attempting to relate the results reported in this Chapter to the binding of effectors under physiological conditions. In a solution of pH 7.1 and a total haemoglobin concentration of approximately 30%, the relative amount of the $\alpha\beta$ unit must be extremely small. The deoxy form of the predominant $(\alpha\beta)_2$ tetramer undoubtedly forms a 1:1 complex with effector (Benesch and Benesch, 1969; Arnone, 1972) but it is uncertain whether this complex has the same detailed tertiary and quaternary structure as the methaemoglobin tetramer complex formed at pH 5.4. On binding the effector, the methaemoglobin may adopt a structure identical to the deoxyhaemoglobin complex, but this would presuppose that oxyhaemoglobin at pH 5.4 is incapable (or less capable) of binding effector, and preliminary sedimentation velocity experiments with ATP and oxyhaemoglobin A have indicated that this is not so. Nevertheless, the present results do strongly support (albeit indirectly) the basic contention that in aqueous solution a 1:1 tetramer-effector complex would be formed at pH 7.1, and on this basis it is possible to make some general comments on oxygen binding under physiological conditions. For this purpose, haemoglobin is considered to exist at pH 7.1 in two conformational states, the oxy- and deoxy-$(\alpha\beta)_2$ forms (Perutz, 1970a, 1972; Arnone, 1972). According to inequality (I-24) a sigmoidal oxygen binding curve (independent of total protein concentration) would arise if the isomerisation constant were greater than 1/3, since the number of oxygen binding sites per mole of tetramer is 4. The organic phosphate effectors ensure the observed "right-shift" (Brewer and Eaton, 1971) by causing X*, defined by

equation (I-54b) to be greater than X. It could be noted that the binding curve for effector itself need not be sigmoidal, and indeed it is evident from Table (I-1) that such a curve, obtained with a fixed but non-saturating oxygen tension would be a rectangular hyperbola, regardless of whether both conformational states, or only one, possessed a single binding site for the effector. The latter possibility is the one proposed by Perutz (1970b) and Benesch, Benesch, Renthal and Gratzer (1971) who claim that the oxy-state cannot bind effector. The former possibility (Garby, Gerber and De Verdier, 1969) implies that both states may bind effector, the deoxy state preferentially. The present data on the effect of ATP on met- and oxyhaemoglobin at pH 5.4 (where the binding constant is larger than at pH 7.1) suggest that this possibility certainly cannot be excluded.

In final summary, it could be noted that the material in this Chapter illustrates the applicability of several findings reported elsewhere in this work. Firstly, it has been shown that differential chromatography (Chapter III) is useful for detecting the preferential binding of a ligand to one polymeric form of an acceptor coexisting in equilibrium with other states. Secondly, it has used the weight-average quantities (Chapter II), elution volumes, sedimentation coefficients and molecular weights to determine the values of binding parameters which could not be readily evaluated through direct binding studies. The theory developed for this purpose was based on the binding equations reported in Chapter I. Thirdly, the theoretical results reported in Chapter I have also been employed, briefly, to

discuss the sigmoidal binding of oxygen to haemoglobin and its "right-shift" on the addition of organic phosphate effectors.

It is hoped that the newly developed methods, and the theory of Chapter I may find general use in similarly elucidating the wide variety of enzyme and protein systems important in metabolic control. With the recognition of the existence of regions of sigmoidality for a given system, it has become clear that a knowledge of the factors affecting the association equilibria of proteins may be critically important to an understanding of the effect of environmental parameters (including total acceptor and effector concentrations) on biologically important control responses.

CHAPTER V

MATERIALS AND METHODS

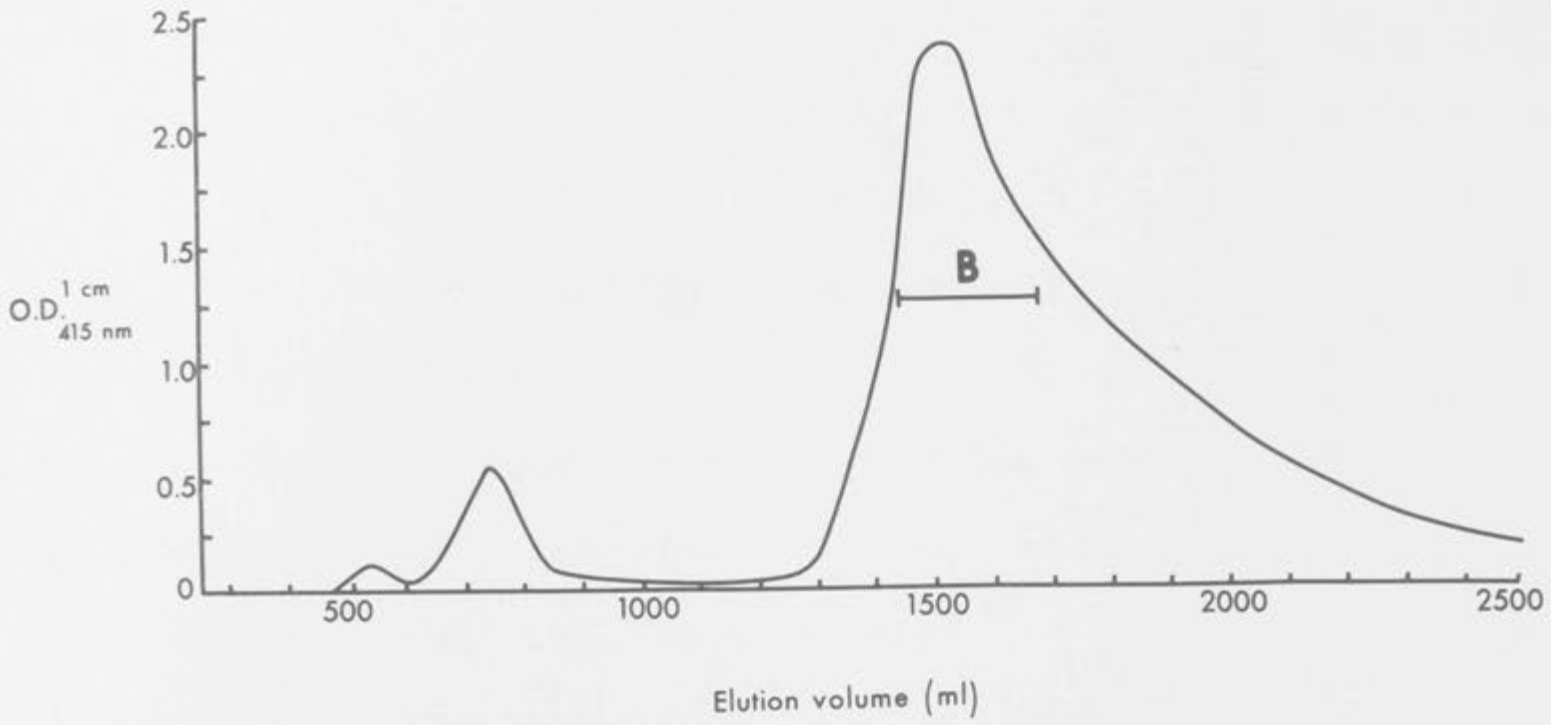
1. Materials.

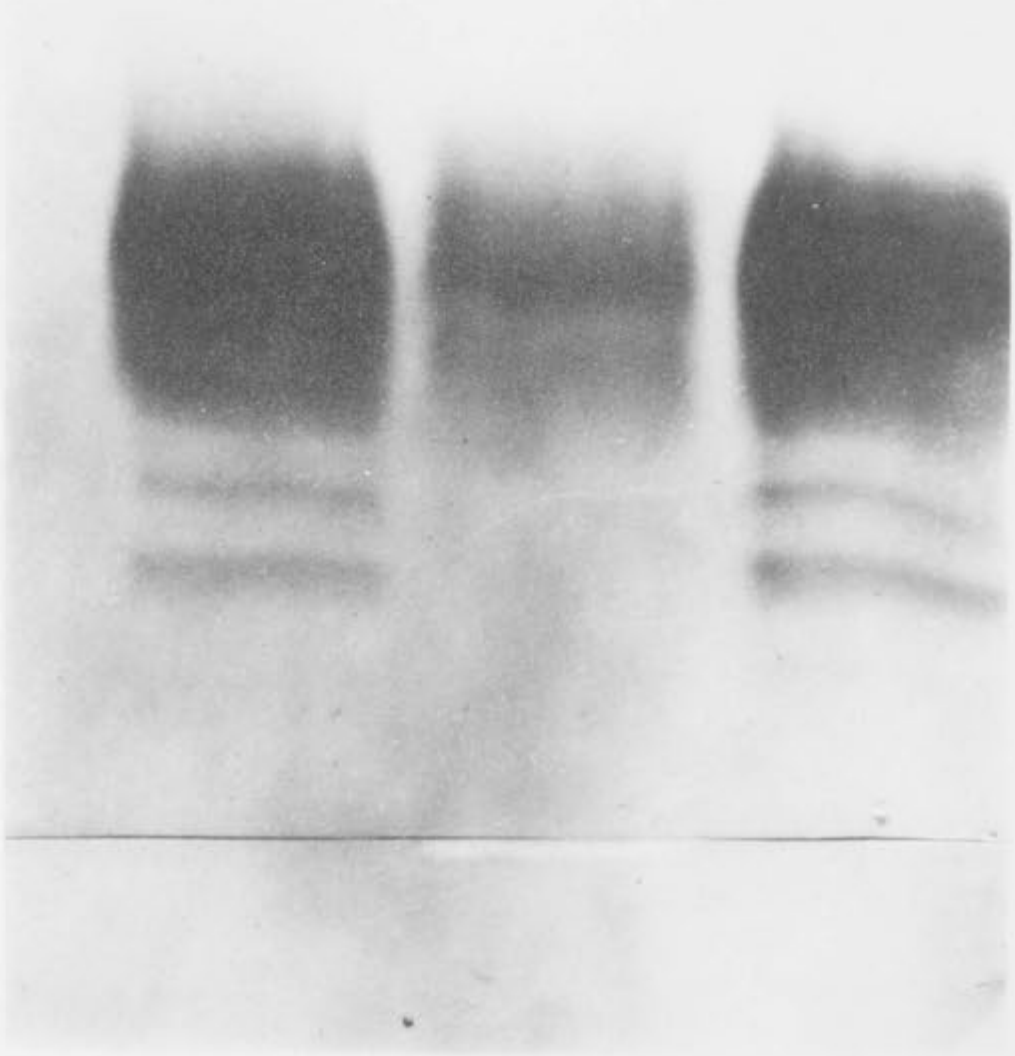
(a) Proteins

(i) β-lactoglobulin. Bovine β-lactoglobulins A and B were prepared from the milk of individual cows which had been typed homozygous for the appropriate variant by starch gel electrophoresis (Bell and McKenzie, 1964; Section 2(b) of this Chapter). The preparative procedure, a modification of that proposed by Aschaffenburg and Drewry (1957), is described in detail under Method 1a of Armstrong, McKenzie and Sawyer (1967). Butterfat and casein were separated from whole milk by the addition, with stirring, of 264 g of solid ammonium sulphate per litre. The mixture was stirred for 2 hours, filtered through Whatman 541 filter paper and acidified to pH 3.5 with 1 M HCl (approximately 50 ml/l whey, added slowly over a period of 40 minutes) in order to precipitate α-lactalbumin. After standing for an hour the precipitate was sedimented by centrifugation for 40 minutes at 12,200×g and the supernatant was titrated back to pH 6 with 1 M ammonia. β-Lactoglobulin was precipitated by the addition of $(NH_4)_2SO_4$ (262 g/l), the mixture being left to stand overnight. The precipitate was collected by centrifugation at 34,800×g for 20 min and redissolved by dialysis against an acetate buffer (16.33 g sodium acetate.$3H_2O$), 2.3 ml glacial acetic acid per litre) at the isoelectric point of β-lactoglobulin, pH 5.2. Crystals obtained by subsequent dialysis against glass distilled water were redissolved in the acetate buffer, pH 5.2, and recrystallised protein was stored in a damp state in the presence of a trace of toluene.

(ii) Pooled haemoglobin, haemoglobin A and its α and ß polypeptide chains. Red blood cells were sedimented from freshly drawn venous blood from a single donor by centrifugation for 10 minutes at 2700 X g. After several washings with cold 0.9% sodium chloride, the cells were lysed with three volumes of glass-distilled water per volume of packed cells, and allowed to stand overnight. Adenosine triphosphate and minor protein contaminants were adsorbed to charcoal (Norite A) and DEAE-Sephadex A50 (Garby, Gerber and De Verdier, 1969), and cell debris were removed by centrifugation at 78,000 X g for 1 hour in a Spinco Model L ultracentrifuge. Following further clarification with 8µ, 3µ and 0.22µ Millipore filters, the solution of pooled haemoglobins was dialysed against a 0.15 M phosphate buffer, pH 6.8, and stored in the cold under carbon monoxide. The final solution generally contained around 4 g/100 ml of pooled carbonmonoxyhaemoglobins. Solutions of higher concentration were prepared by concentrating the Millipore filtrate with a Diaflo ultrafiltration unit prior to dialysis against the phosphate buffer.

Haemoglobin A was separated from the other variants by ion-exchange chromatography (Hill, Konigsberg, Guidotti and Craig, 1962) on Bio-Rex 70, 200-400 wet mesh, a resin with carboxylic acid exchange groups supplied by Bio-Rad Laboratories. The resin was pretreated according to the procedure outlined by Hill *et al.* (1962) and equilibrated with a 0.15 M phosphate buffer pH 6.4. A typical elution profile together with the results of starch gel electrophoresis of pooled haemoglobin and the cut taken for


2.5
2.0
1.5
1.0
0.5
0
O.D. 1 cm 415 nm
B
500
1000
1500
2000
2500
Elution volume (ml)



A B A

FIGURE V-1

The fractionation of pooled haemoglobins on the cation exchanger Bio-Rex 70. (a) An elution profile obtained by loading 200 mg of protein in 10 ml onto a 36 cm x 5 cm column of the exchange resin. The protein was eluted with a 0.15M phosphate buffer, pH 6.4, at the rate of 18-20 ml/hr. (b) A comparison of starch gel electrophoresis results obtained with the original sample (A) and the fraction ($\overset{B}{\vdash\!\!\!-\!\!\!\dashv}$) taken to be haemoglobin A.

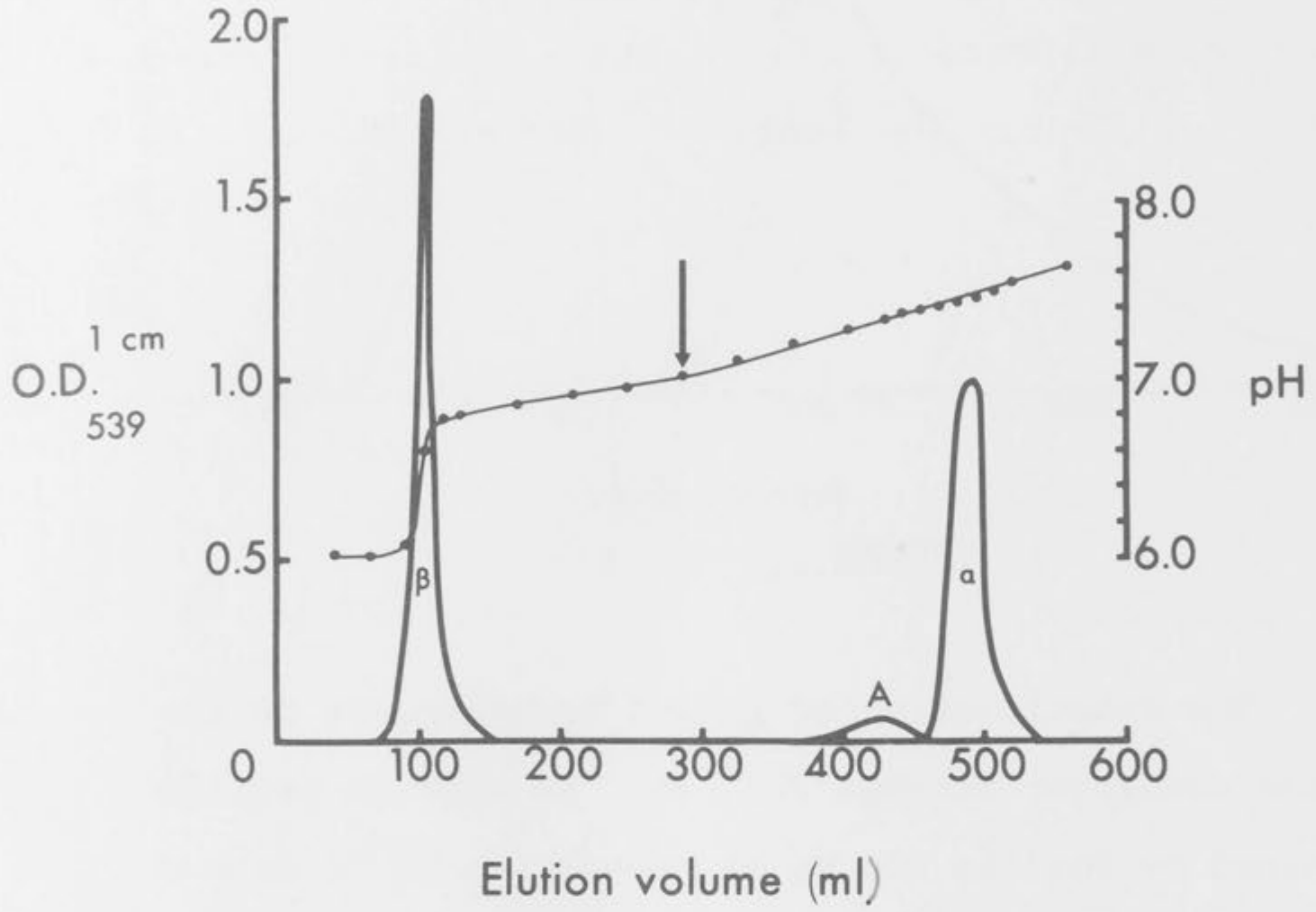


FIGURE V-2

The fractionation of α and β chain mercuribenzoate derivatives on carboxymethyl cellulose. Approximately 100 mg of protein in 5 ml were applied to a 10 cm x 2.5 cm column of Whatman carboxymethyl cellulose equilibrated with 0.02 M phosphate buffer pH 6.0. Details of the method used to generate the pH gradient (●—●) are given in the text. The vertical arrow indicates the point at which the volumes of the buffer reservoirs were reduced in order to produce a steeper gradient.

use in subsequent experiments is shown in Figure (V-1).

The α and β chains of the purified haemoglobin A were dissociated by reacting their sulphydryl groups with p-chloromercuribenzoate in a solution of ionic strength 0.1, pH 6, according to the method of Bucci and Fronticelli (1965). The mercuribenzoate derivatives were then separated by gradient elution from a 2 x 10 cm column of Whatman carboxymethyl cellulose. Bucci and Fronticelli (1965) generated the pH gradient with two bottles of equal size, one containing 1 litre of 0.01 M phosphate buffer, pH 6.7 and the other containing 1 litre of 0.02 M K_2HPO_4, the former being connected directly to the column. This procedure was hastened considerably, without loss of resolution, by transferring the two solutions to 250 ml flasks in order to produce a steeper gradient after the β-chains had been eluted (Figure V-2). Finally, the sulphydryl groups in the two chains were regenerated by rapid emulsification of the aqueous solutions of the mercuribenzoate derivatives with 1-dodecanethiol (De Renzo *et al.*, 1967). The success of the regeneration was judged by the ability of β-chains to reassociate and form haemoglobin H(β_4) with a sedimentation coefficient of 4.7, and by the ability of equimolar amounts of α and β chains to associate and produce a species electrophoretically indistinguishable from haemoglobin A.

All the experimental results obtained with haemoglobin A at concentrations below 10 g/l were performed with the phosphate free met derivative. Potassium dichromate (10 moles per mole of haem) was used to oxidize the haem-iron, and the excess reagent, together with phosphate ions, was

removed by dialysis against distilled water followed by passage through a 10 cm x 2.0 cm column of Dowex I-X2 anion exchange resin in the chloride form.

(b) Buffers and other chemicals

Aqueous buffers with compositions reported in the text were prepared from glass distilled water and analytical grade reagents supplied by British Drug Houses Ltd., Hopkins and Williams Ltd., and Ajax Chemicals Ltd. The pH values of buffers were measured at the appropriate temperature using a Radiometer pH meter (model 26) equipped with glass and calomel electrodes. The meter was routinely calibrated with standard buffer solutions immediately before a measurement. Deuterium oxide (99.8 atom %) was obtained from International Chemical and Nuclear Corp. and used without further treatment. Buffer salts were dissolved directly in the D_2O and the pD was measured using the pH meter as described in Chapter II.

Poly-L-glutamic acid supplied by Sigma Chemical Co. was reported to have a weight-average molecular weight, M_w of 102,000. This was confirmed by the ultracentrifugal method of Archibald (1947) as described by Klainer and Kegeles (1955). A value of 104,000 ± 5,000 for M_w was recorded in the pH 4.8 buffer and a value of 99,000 ± 5,000 at pH 5.4. Solutions were prepared by dissolving the dry poly-L-glutamic acid in a solution of sodium acetate and titrating it to the desired pH with acetic acid.

Organic phosphates: Crystalline equine adenosine-5'-triphosphate (disodium salt) was obtained from Sigma Chemical Co., and the barium salt of 2,3-diphospho-D-glyceric

acid was supplied in 100 mg lots by Calbiochem. The latter compound was dissolved by shaking with a few grams of Zeo Karb 225 cation-exchange resin in the sodium form. The resin was then washed with 5 x 10 ml of glass distilled water and the washings were collected and freeze-dried to recover the sodium salt of 2,3-DPG.

Total phosphate in either ATP or 2,3-DPG solutions was determined by estimating inorganic phosphate after hydrolysis in boiling 1 N HCl for 20 minutes (Ames and Dubin, 1960). The assay procedure for inorganic phosphate involves the formation, and subsequent reduction with ascorbic acid, of a phosphomolybdate complex (Chen, Toribara and Warner, 1956), the optical density of the final solution at 820 nm providing a direct measure of the initial quantity of inorganic phosphate in the assay mixture. Phosphate as ATP or 2,3-DPG was found by subtracting the inorganic phosphate content of unhydrolysed solutions from the total.

2. Methods.

(a) General laboratory methods

All solutions except those of haemoglobin and β-lactoglobulin were prepared by dissolving a known weight of the dry solute in the appropriate solvent. Weighings were made with a Mettler H10T balance, and solutions were adjusted to their final volumes in volumetric flasks. Since haemoglobin was stored as a concentrated solution and β-lactoglobulin in the form of damp crystals, protein concentrations were determined spectrophotometrically using a Zeiss (model PMQ II) spectrophotometer, and known values for the extinction coefficients ($E^{1\%}_{1\ cm}$). A value of 9.6 at

278 nm was used for estimating β-lactoglobulin (McKenzie, Sawyer and Treacy, cited by Bell and McKenzie, 1967). By a suitable selection of light pathlength and wavelength, it was possible to use the absorption bands of haem groups to estimate haemoglobin concentrations over a wide range without dilution. Values of $E^{1\%}_{1\ cm}$ at nine different wavelengths for each particular liganded state of haemoglobin were calculated from the molar extinction coefficients reported by Benesch, Macduff and Benesch (1965) and Lemberg and Legge (1949). All solutions were passed through a washed 0.45μ Millipore filter immediately prior to the measurement of optical densities.

Dialyses were performed at 4° with Visking 8/32 cellophane tubing which had been washed by immersion in hot 3% acetic acid for at least 4 hours, followed by thorough rinsing in glass-distilled water. The minimum time of dialysis was 24 hours with two changes of buffer at 4 and 8 hours, and in most cases there were at least 100 volumes of buffer to 1 volume of protein solution. To minimise acid hydrolysis of ATP in the solutions used in the sedimentation equilibrium experiments reported in Chapter IV, methaemoglobin was first dialysed for 19 hours against the acetate buffer of pH 5.4 in the absence of ATP. ATP was then added to both the protein solution and a reduced volume of dialysate, in amounts chosen to achieve equal concentrations of the ligand in both solutions, and the dialysis was continued for a further five hours.

(b) Starch gel electrophoresis

Horizontal starch gel electrophoresis (Smithies, 1955) was performed at 20° using a thin starch gel slab (20 x 15.5 x 0.15 cm) prepared by the method of McKenzie (1971). For the identification of β-lactoglobulin variants the gel was made from 13-15 g of starch (Connaught Laboratories) dissolved in 100 ml of buffer containing 0.028 M boric acid and 0.012 M NaOH, pH 8.5, and the same buffer was used in the electrode vessels (Bell and McKenzie, 1964). Electrophoresis of haemoglobin variants was carried out in a discontinuous system of buffers. The gel was prepared using the same quantity of starch in 100 ml of 0.076 M *Tris*-(hydroxymethyl)-aminomethane, 0.005 M citric acid, pH 8.65, and the electrode vessels contained a solution of 0.3 M boric acid and 0.05 M NaOH (Poulik, 1957).

Strips of Whatman 3MM filter paper were impregnated with samples and inserted in slots in the gel. Electrical contact between the gel and electrode solutions was made with cellulose sponge strips, and a constant voltage of 150-180 V (7 V/cm) was applied with a DC power supply for 4-5 hours. After electrophoresis, the gel was inverted and immersed in dye solution (3 volumes of nigrosine, 0.1% (w/v) in methanol, 2 volumes of glacial acetic acid, and 1 volume of water). After staining for 15 minutes the gel was drained of dye and washed in a solution of methanol, glacial acetic acid and water 5:5:1 (by volume).

(c) Column procedures

All column chromatography experiments were performed with jacketed glass columns supplied by Scientific & Research Equipment Co. (Aust.) and Chromatronix Incorporated.

Temperature control was achieved by circulating water through the jacket from an Aminco or Colora thermostatted bath. Sephadex beads from Pharmacia Fine Chemicals were equilibrated with buffer for the times recommended by the manufacturer (1-3 days), the buffer being changed periodically by decantation. Sufficient buffer was added to the pre-equilibrated Sephadex so that 2.5 volumes of the mixture contained approximately 1 volume of swollen beads, and immediately prior to packing the column the suspension was degassed by rapid swirling under vacuum. The column was filled to one-third of its height with buffer, the slurry was introduced, and then the column was opened to allow a steady flow of buffer. This flow was maintained by means of a Beckman metering pump and a minimum time of 18 hours was allowed for bead packing. Temperature control and a constant flow of buffer were maintained at all times between experiments.

In experiments with β-lactoglobulin A, a known volume of protein solution was added to the column from a glass syringe, ensuring that the head of liquid above the Sephadex was never greater than 1 cm. In other experiments it was found convenient to pump the protein solution on to the column by means of the simple displacement apparatus shown in Figure (V-3). This arrangement allowed much better control of flow rate (0.1 - 0.5 ml/min.) and the total weight of solution loaded on to the column was determined simply by the difference in the weights of the flask containing the protein solution before and after the experiment. As soon as loading had begun, small aliquots of the eluate were collected, manually, in pre-tared tubes,

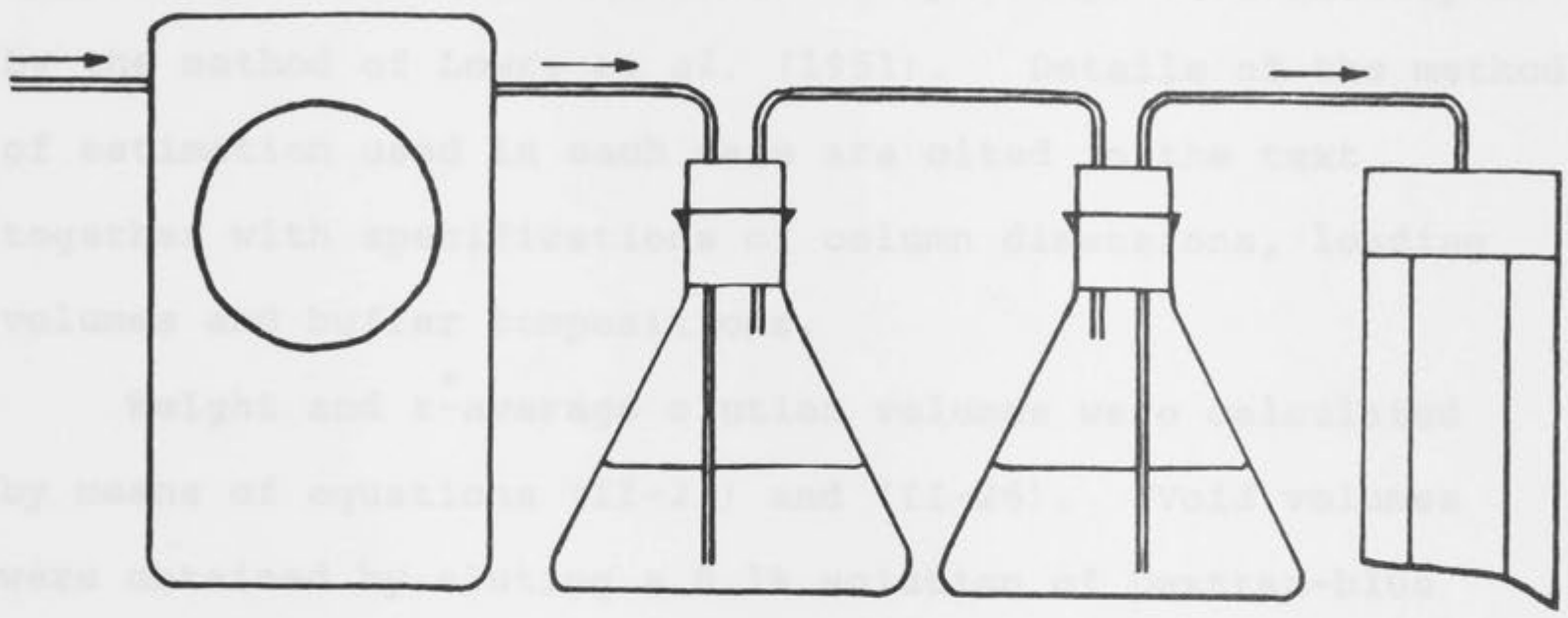


FIGURE (V-3)

A schematic diagram of the displacement apparatus used to pump protein solutions on to Sephadex columns during frontal gel chromatography experiments.

at the exit point of the column. The 'dead-space' of Chromatronix columns was less than 0.1 ml. When loading was complete, the protein was eluted by pumping buffer directly on to the column. The weight of each aliquot was determined, and divided by a density term to give the corresponding volume. The concentration of the protein in each aliquot was determined either spectrophotometrically or by the method of Lowry *et al.* (1951). Details of the method of estimation used in each case are cited in the text together with specifications of column dimensions, loading volumes and buffer compositions.

Weight and z^*-average elution volumes were calculated by means of equations (II-23) and (II-26). Void volumes were obtained by eluting a 0.1% solution of Dextran-blue (M_w 2 x 10^6, Sigma) and monitoring the eluate at 620 nm. The low concentration of dextran blue was selected to minimise osmotic effects.

(d) Optical rotatory dispersion measurements

A Perkin-Elmer model 141 polarimeter was used to measure the rotation of plane-polarised light at wavelengths of 365 nm, 436 nm, 546 nm, 578 nm and 589 nm (Melbourne), or 313 nm, 364 nm, 436 nm, 546 nm and 579 nm (Canberra). Readings were reproducible to within 0.002°. A quartz cell with a capacity of 1 ml and a pathlength of 10 cm was employed, and the temperature was controlled to ± 0.1° by circulating water from an Aminco thermostatted bath through the cell jacket. All solutions were filtered through 0.45μ Millipore filters prior to ORD measurement, and readings were corrected for the small blank readings obtained with

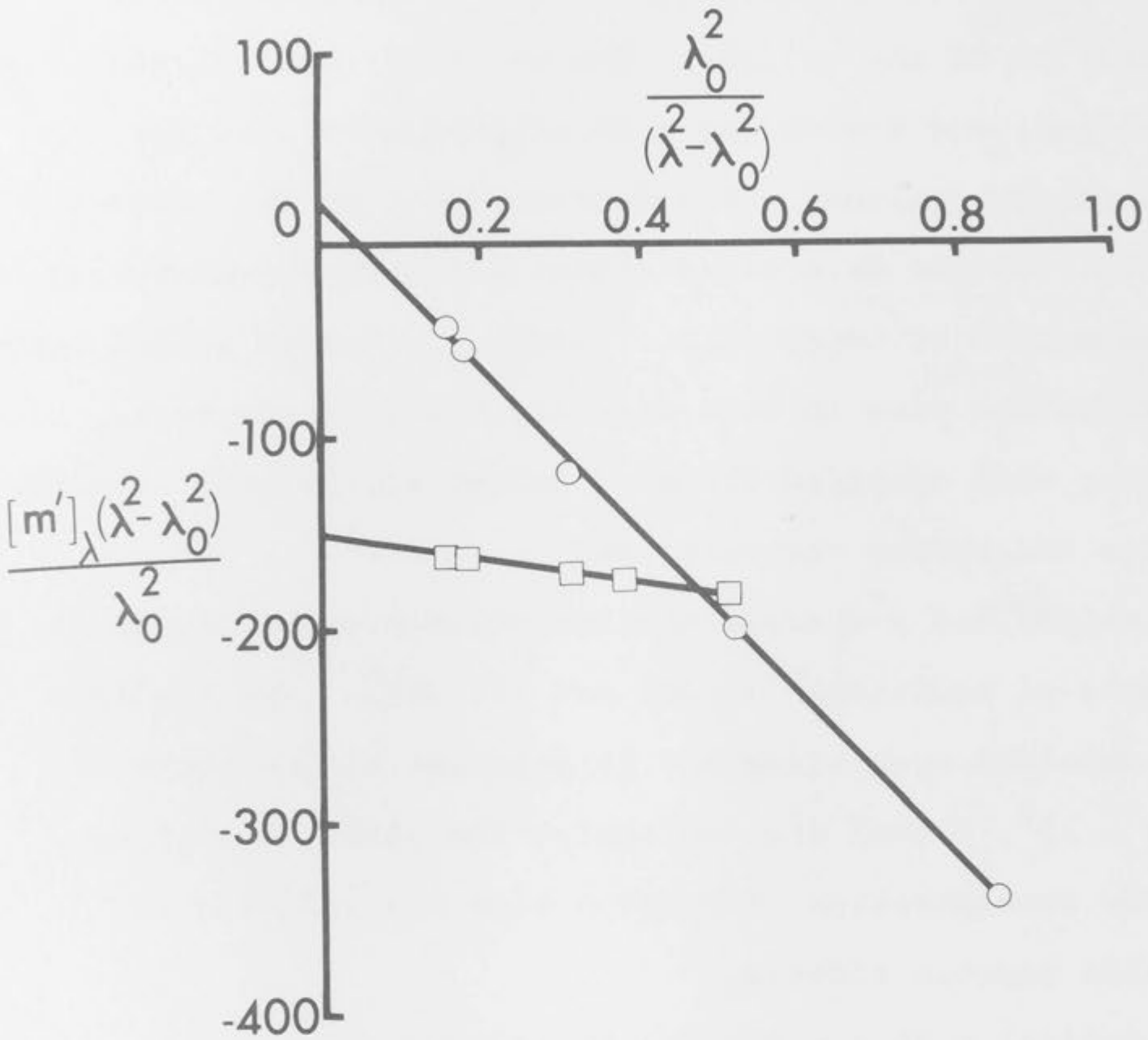


FIGURE V-4

Representative Moffitt-Yang plots obtained with bovine β-lactoglobulin A (□), (13 g/l, dissolved in 0.1 M sodium acetate, pH 4.7) and poly-L-glutamic acid (O) (5 g/l, dissolved in 0.13 M sodium chloride, 0.07 M sodium acetate, pH 4.90). Optical rotations were measured, in each instance, at 20°.

solvent alone. A mean residue rotation, $[m]_\lambda$, expressing the observed rotation, α_λ, per mean residue weight (MRW) and per unit pathlength, d, was calculated according to the definition

$$[m]_\lambda = MRW\alpha_\lambda/cd \qquad (V\text{-}1)$$

where c is the weight concentration (g/ml) of the protein or polypeptide. The mean residue weight of β-lactoglobulin was taken to be 110 while that of polyglutamic acid was 129. Values of $[m]_\lambda$ were then corrected to *reduced mean residue rotations* $[m']_\lambda$ by the Lorentz factor $3/(n_\lambda^2 + 2)$ where n_λ is the refractive index of the solvent (Fasman, 1963). The data $[m']_\lambda$ *vs.* λ were fitted by the two-term phenomenological equation of Moffitt and Yang (1956):

$$[m']_\lambda = a_o\lambda_o^2/(\lambda^2-\lambda_o^2) + b_o\lambda_o^4/(\lambda^2-\lambda_o^2)^2 \qquad (V\text{-}2)$$

where a_o and b_o are constants for a given protein in a fixed environment, and λ_o, taken to be 212 nm is an empirical constant related to the resonant frequencies (absorption bands) of the electrons responsible for optical rotations at much higher wavelengths. It can be seen that if the observed wavelength dispersion of optical rotations obeys the Moffitt-Yang equation, a plot of $[m']_\lambda.(\lambda^2-\lambda_o^2)/\lambda_o^2$ *vs.* $\lambda_o^2/(\lambda^2-\lambda_o^2)$ will be linear with slope b_o and ordinate intercept a_o. Representative plots are shown in Figure (V-4) from which it is clear that equation (V-2) adequately describes the data recorded for both β-lactoglobulin and poly-L-glutamic acid.

Estimates of helical content were obtained by dividing the observed b_o value by -580, a value recommended by Chen and Yang (1971) on the basis of X-ray data on several proteins: previously a value of -630 had been suggested on the basis of measurements made with homopolypeptides. Variations of a_o values have been interpreted in terms of alterations in local environment of particular residues, including solvent effects (Urnes and Doty, 1961): their use as weight-average quantities is discussed in Chapter II.

(e) Ultracentrifuge methods

All experiments were performed with a Spinco Model E analytical ultracentrifuge. The machine used for the sedimentation velocity experiments with β-lactoglobulin A was equipped with a mechanical speed control; but experiments with haemoglobin were performed with an ultracentrifuge fitted with an electronic speed control. Aluminium An-D or titanium An-H rotors were used and the temperature was controlled to within 0.1°C by the RTIC unit. Double-sector, 12 mm filled-epon centrepieces and sapphire windows were used for all equilibrium experiments, and for the sedimentation velocity of the α and β chains of haemoglobin A. Other sedimentation velocity experiments were performed with aluminium 12 mm single-sector centrepieces and quartz windows. A + 1° wedge-window was used when it was desired to compare directly two sedimentation velocity experiments. The light source was a mercury AH6 lamp, and the filters and photographic plate-types used are shown in table form as follows:

protein	experimental design	Wratten filter	Kodak photographic plate
β-lactoglobulin A	sedimentation velocity	77A	metallographic
haem protein	sedimentation velocity	B29	I-N
haem protein	sedimentation equilibrium	77A	II-G

(ii) Sedimentation velocity. The photographic record of the schlieren pattern (a plot of refractive index gradient *vs.* radial distance) was aligned on a two-dimensional comparator (Nikon Shadowgraph or Gaertner Scientific Corporation) and the radial distance $X_R - X_P$ from the schlieren peak at X_P to the reference edge, X_R at 7.30 cm from the axis of rotation, was measured. Division of this distance by the horizontal magnification factor and subtraction from 7.30 cm gave the distance, x_p, from the axis of rotation to the maximum ordinate of the peak in the cell. The position of the meniscus in the cell, x_m was also obtained in the same manner. Values of x_m and x_p were determined for several photographs taken at different times during the experiment, a constant value of x_m providing an assurance that no material had leaked from the cell during the run. At temperature T, in buffer b, the sedimentation coefficient, $s_{T,b}$, defined as the rate of movement of x_p per unit gravitational field, was determined by least-squares linear regression of $\ln x_p$ on time t. In detail

$$s_{T,b} = \frac{dx_p/dt}{x_p\omega^2} = \frac{d\ln x_p/dt}{\omega^2} \qquad (V\text{-}3)$$

where ω is the angular velocity in radians per second. In order to place values of $s_{T,b}$ on a common basis for comparison,

they were corrected to hypothetical sedimentation coefficients $s_{20,w}$, at 20° in water by the procedure outlined in Chapter II. Physical constants for aqueous buffers were taken from the International Critical Tables while those for deuterium oxide were drawn from the tables of Kirschenbaum (1951).

Weight-average sedimentation coefficients, $\bar{s}_{T,b}$ were determined by means of equation (V-3) with x_p replaced by $\bar{x}_p$, the position corresponding to the square root of the second moment of the entire schlieren peak. Goldberg (1953) defined $\bar{x}_p$ as follows

$$\bar{x}_p^2 = \frac{\int_{x_m}^{x_p} x^2 dn/dx.dx}{\int_{x_m}^{x_p} dn/dx.dx} \simeq \frac{\sum_{i=1}^{n} x_i^2 (dn/dx)_i}{\sum_{i=1}^{n} (dn/dx)_i} \qquad \text{(V-4)}$$

where x_p refers to any radial position in the solution plateau and (dn/dx) is the refractive index gradient of the solution at the corresponding x. Values of $(dn/dx)_i$ were measured, in practice, as the height of the schlieren pattern above the baseline at n equally spaced values, x_i, across the entire boundary (regardless of its shape) and the integrals were estimated numerically by the trapezoidal integration formulae indicated in equation (V-4).

Bimodal reaction boundaries were analysed, not only in terms of $\bar{s}$ but also in terms of $\bar{c}_{min}$ (equation II-20), the concentration corresponding to the back peak area. Using the recorded values of dn/dx_i *vs.* x_i the boundary was drawn

on squared paper and resolved manually into a front and back peak, the area of the latter being determined by planimetry.

(ii) Sedimentation equilibrium of methaemoglobin A.

The Rayleigh interference optical system with offset limiting aperture was used in all experiments. Initial concentrations of protein solutions were determined in separate experiments using a 12 mm. double sector synthetic boundary cell with a capillary between the two sectors. In one sector was placed 0.14 ml of dialysed protein solution, and in the other 0.44 ml of dialysate. The rotor was accelerated to 10,000 r.p.m. and as soon as the menisci were superimposed, the rapid brake was applied and the speed was reduced to 6,000 r.p.m. Once the latter speed had been reached a photograph was taken, and a second was taken when sufficient diffusion had occurred for the fringes to be resolved in the boundary region. The whole number of fringes was determined from the second photograph, and the fringe fraction from the first. When the rotor had come to rest it was removed from the chamber, shaken to destroy the concentration gradient, and returned to 6,000 r.p.m. for a baseline photograph. Following the procedure of Richards, Teller and Schachman (1968) the baseline was used to make small corrections to the concentration J_o (in fringes) determined from the first two photographs.

For the equilibrium centrifugation a small amount of 1,3-butanediol was added to the dialysate in order to raise the refractive index of the reference liquid to that of the protein solution, and hence improve the clarity of the interference pattern by bringing the fringes closer to the

zero-order or achromatic fringe (Richards and Schachman, 1959). To assist in the location of the base of the solution column, x_b, and to provide an arc-shaped base, 0.01 ml of the inert fluorocarbon FC43 was placed in the bottom of each cell compartment. A 3 mm solution column was obtained with 0.11 g of solution, the reference channel being filled with 0.12 g of dialysate. The equilibrium speed was determined by the procedure of Richards *et al.* (1968) using an arbitrarily assigned maximum desirable gradient at the base of the solution column of 100 fringes/cm. The time to reach equilibrium was reduced by the overspeeding technique of Howlett and Nichol (1972b), but since there was some doubt concerning the choice of diffusion coefficient, centrifugation was always continued for 24-28 hours, there being no measurable change in the interference patterns after this time. At the completion of the run, the concentration gradient was destroyed by shaking the rotor, which was then returned to the equilibrium speed for a baseline photograph.

Interference patterns were measured according to the procedure of Richards *et al.* (1968). The final baseline photograph was aligned on the comparator, the radial positions of the inner and outer reference edges were determined, and the deviations of the nearest fringe from an arbitrarily chosen level parallel to the line joining the centre reference fringes were measured at small intervals of x throughout the entire solution column. These deviations were plotted against x and a smooth curve was drawn through them to obtain the cell-deviation plot. Next, the equilibrium pattern was traversed at the same level as

that chosen for the baseline, and the radial positions at which a fringe was crossed, were recorded. The positions of the meniscus and cell bottom were also recorded. The procedure of correcting for the baseline was made considerably easier if the positions of the inner and outer reference edges on the comparator were the same for both the equilibrium and baseline photographs. Deviations of the baseline at each fringe position in the equilibrium traverse were found by interpolation.

Apparent weight-average molecular weights were calculated from the fringe data with a Univac 1108 digital computer and a programme based on the flow chart of Richards *et al.* (1968). Input to the programme consisted chiefly of comparator readings for the inner and outer reference positions of the equilibrium pattern, the meniscus and bottom of the solution column, a vector of fringe locations followed by a vector of corresponding baseline corrections, and the initial concentration, J_o, in fringe units. The concentration c_m at the meniscus, x_m was determined by application of the conservation of mass equation

$$J_o - J_m = \frac{(J_b - J_m)x_b^2 - \int_{J_m}^{J_b} x^2 dJ}{x_b^2 - x_m^2} \qquad (V-5)$$

where J_b is the concentration at the cell base. The difference $J_b - J_m$ was approximated by the total number of fringes crossed in the equilibrium photograph plus the small increments to account for the concentration gradient between

the meniscus and the first fringe, and between the last fringe and the base of the solution column (Richards *et al.*, 1968). Once the approximate concentration at the meniscus was known, all the other fringe locations were assigned absolute concentrations (fringe-labelling), with small adjustments to account for the cell deviation, and curve-fitted by orthogonal polynomial regression to provide better estimates of J_b, J_m, and hence $J_o - J_m$. The fringes were then relabelled and curve-fitted to provide a still better estimate of $J_o - J_m$, this procedure being repeated until the estimates converged (usually in 3 or 4 iterations) to a constant value. From the manner in which the patterns were measured, one fringe position should have been labelled with the initial concentration (the hinge-point). Due to difficulties in locating precisely x_m and x_b, there was sometimes a discrepancy of as much as 0.3 fringe between J_o and the nearest fringe label. In such circumstances, it was decided to relabel this nearest fringe, J_o, and make appropriate adjustments to the other fringe labels. Direct use of J *vs.* x data was made in calculating X* in Chapter IV, but apparent weight-average molecular weight *vs.* c curves were obtained in the following way. $\ln J$ *vs.* x^2 data were fitted with a polynomial of the form

$$\ln J = \sum_{i=o}^{p} \beta_i x^{2i} \qquad \text{(V-6)}$$

The coefficients, β_i, were estimated by the method of multiple linear regression, a quadratic polynomial (p = 2) being sufficient to describe the observed data in most cases.

Values of $d\ln J/dr^2$ ($= \sum_{i=1}^{p} i\beta_i x^{2(i-1)}$) were then calculated and substituted in equation (IV-21) to give $M_w(r)_{app}$ as a function of J. Concentrations of methaemoglobin in fringes were converted, if it was so desired, to the more familiar weight concentration scale (g/l) by division by 4.50 (Babul and Stellwagen, 1969).

(f) Computations

The computer programme for the analysis of sedimentation equilibrium data which is described in detail in the previous section was based on an earlier programme also written by the present author (Baghurst, 1969). Since it has been acknowledged in other work (Ashman and Atwell, 1972; Howlett *et al.*, 1972) the code (in Fortran IV) is listed in Appendix II in the hope that it may find general use.

Many other smaller calculations were performed routinely with a PDP-8/l digital computer using a programming language called FOCAL. These included the calculation of theoretical binding curves in Chapter I, mass migration profiles in Chapters II and III, weight-average sedimentation coefficients, $\bar{s}$, in Chapter II, and apparent association constants X* and sedimentation equilibrium distributions in Chapter IV.

ABSTRACT

The work commences with the development of equations which describe the binding of a small molecular weight ligand to a self-interacting protein acceptor (either isomerising or polymerising). It is shown that preferential binding of the ligand to one state of the acceptor is a necessary, but not sufficient condition to ensure a sigmoidal response, which is defined by the occurrence of a point of inflection in the binding curve. The boundaries of regions within which a given system will exhibit sigmoidality are defined in terms of numbers of binding sites, relevant equilibrium constants and (in the case of a polymerising acceptor), the total acceptor concentration. These regions are considered in relation to loci of values of the equilibrium constant governing the self-interaction of the acceptor at different temperatures and pressures to emphasize the necessity of considering variations in environmental parameters in relation to the changing forms of sigmoidal responses. Chapter I concludes by stressing the necessity of investigating the factors which affect association-dissociation equilibria.

Chapter II is concerned with factors which affect the extent of association of a genetic variant (A) of bovine β-lactoglobulin. Particular attention is given to the effect of transferring the protein from aqueous to deuterium oxide media. It is shown by the use of weight-average quantities (elution volumes, sedimentation coefficients and optical rotatory dispersion parameters) that the extent of association is increased in deuterium

oxide. Estimation of the enthalpy and entropy changes involved, leads to the conclusion that an increased association in deuterium oxide cannot be used as the sole criterion for the detection of intermolecular hydrophobic interactions, as had previously been suggested.

In Chapter III, the same self-interacting system β-lactoglobulin A is used to investigate the possibility of employing differential chromatography to study the effect of environmental parameters on the extent of association. The method is then applied to an investigation of the action of effectors on the extent of association of pooled adult human methaemoglobin at pH 5.4. It was pointed out in Chapter I that the binding of these effectors may well determine the form of the binding curve, obtained with a dissimilar ligand, through their effects on the apparent association-dissociation equilibrium of the acceptor.

The preferential binding of the organic phosphate effectors, adenosine 5'-triphosphate (ATP) and 2,3-diphospho-D-glycerate (DPG) is further investigated in Chapter IV by the methods of sedimentation velocity and sedimentation equilibrium. Theories required to interpret the results are derived on the basis of binding equations developed in Chapter I, and on equations describing the mass migration of interacting systems, which are reviewed in Chapter II. The findings show that one molecule of organic phosphate effector binds per molecule of methaemoglobin tetramer (two α chains and two β chains) and that the dimer also binds effector at one site, but with a reduced affinity. In agreement with previous work, it is also reported that

DPG binds more effectively than ATP, and that the binding site on the tetramer involves both β chains. These results are discussed with reference to the claim that the oxy-form of haemoglobin does not bind effector under physiological conditions.

In conclusion, it is postulated that the methods and theories developed in this work may find application in the investigation of a variety of systems in which the preferential binding of ligands is conceivably responsible for the biologically important sigmoidal response.

APPENDIX I

The algebraic solution of Figure (III-5)

In the migration of a system comprising A, C, E, AE and CE ($n = 1$, $p = 1$, $q = 1$), the total mass of both acceptor and modifier must be preserved. The former condition is stated in equation (III-17), while the latter is formulated as follows:

$$\bar{c}_E = M_E\{[E] + [AE] + [CE]\} \tag{1a}$$

$$\bar{v}_E\bar{c}_E = M_E\{v_E[E] + v_A[AE] + v_C[CE]\} \tag{1b}$$

Conservation of mass is stated by $ud\bar{c}_E - d\bar{v}_E\bar{c}_E = 0$, i.e.

$$(u-v_E)d[E] + (u-v_A)d[AE] + (u-v_C)d[CE] = 0 \tag{2}$$

Since $N_A = N_C = [AE]/[A][E] = [CE]/[C][E]$ and $X = [C]/[A]$, equation (2) may be rewritten as

$$(u-v_E) + \{N_A(u-v_A) + N_AX(u-v_C)\}\{[A] + [E]\frac{d[A]}{d[E]}\} = 0 \tag{3}$$

provided $d[E] \neq 0$, a condition which is certainly satisfied in the spread reaction boundary on the advancing side (Figure III-5). At all points in this boundary, it has been shown that $d[\bar{A}] = 0$, where $[\bar{A}] = [A] + N_A[A][E]$. Thus, $d[A]/d[E] = -N_A[A]/(1+N_A[E])$, which may be substituted into equation (3) to yield

$$u = \frac{v_E(1+N_A[E]) + v_AN_A[A] + v_CN_AX[A]}{1+N_A[E] + N_A[A] + N_AX[A]} \tag{4}$$

The value of u' indicated in Figure (III-5) is obtained from equation (4) by setting [E] = 0 and $[A] = \bar{c}_A^0/M_A(1+X)$. Moreover, in general, from equation (1a) and (III-15)

$$[E] = \bar{c}_E/M_E(1+N_A[A]+N_AX[A]) \tag{5}$$

$$[A] = \bar{c}_A/M_A(1+N_A[E]+N_AX[E]+X) \tag{6}$$

Combination of equations (5) and (6) yields the quadratic,

$$[A]^2M_EM_AN_A(1+X)^2 + [A](1+X)(M_AM_E+M_AN_A\bar{c}_E-M_EN_A\bar{c}_A)-M_E\bar{c}_A = 0 \tag{7}$$

To find u^0, the values $\bar{c}_A^0$ and $\bar{c}_E^0$ are substituted into equation (7) which is solved for the positive root $[A]^0$. The corresponding values of $[E]^0$ and u^0 are obtained from equations (5) and (4), respectively. The same procedure is used to construct the gradients between u' and u^0, by varying $\bar{c}_E$ between 0 and $\bar{c}_E^0$ holding $\bar{c}_A$ constant ($d[\bar{A}] = 0$).

It remains to be shown that d[E] = 0 when $u = (v_A+v_CX)/(1+X)$ on the trailing side. Equation (2), which necessarily applies at this point, may be written:-

$$(u-v_E)d[E] + \{(u-v_A) + X(u-v_C)\}d[AE] = 0 \tag{8}$$

The coefficient of the second term in equation (8) is zero when $u = v_A+v_CX/(1+X)$ and the equation reduces to $(u-v_E)d[E] = 0$; the only solution being d[E] = 0, since the value of u under discussion lies between v_A and v_C, both of which are greater than v_E.

APPENDIX II

Computer programme (with sample output) for the analysis of sedimentation equilibrium data.

```
00100     1*      C*****************************************************************
00100     2*      C     FOR THE CALCULATION OF THE APPARENT MOLECULAR WEIGHT FROM ULTRA-
00100     3*      C     CENTRIFUGE SEDIMENTATION EQUILIBRIUM DATA.
00100     4*      C                                          D LN C
00100     5*      C                                 2.R1.T.--------
00100     6*      C                    APP                  D RSQ
00100     7*      C                   M    =   ----------------------
00100     8*      C                     W,R                      2
00100     9*      C                              (1 - VBAR.RHO).OMEGA
00100    10*      C
00100    11*      C     WHERE    R1(GAS CONSTANT) = 8.31432E07
00100    12*      C              T = ABSOLUTE TEMPERATURE
00100    13*      C              C = CONCENTRATION (IN FRINGES)
00100    14*      C              R = RADIUS (CM)
00100    15*      C              RSQ = RADIUS SQUARED
00100    16*      C              VBAR = PARTIAL SPECIFIC VOLUME OF SOLUTE (CC/GM)
00100    17*      C              OMEGA = ANGULAR VELOCITY (RADIANS/SEC)
00100    18*      C*****************************************************************
00100    19*      C
00101    20*            DIMENSION  BUFFER(20),   B1(11,11),   C(100),      DEV(100),
00101    21*           *DSPLCE(100), DSPLC1(100), FLOGC(100),  LOGC(100),   MWRAPP(100),
00101    22*           *P(10),       R(100),      R1(100),     RC(10,50),   RCM (100),
00101    23*           *R1(10),      R0(10),      RSORCE(16),  RSQ(100),    SEB(12,12),
00101    24*           *SS(10),      VSORCE(16),  TITLE(20)
00103    25*            REAL LOGC,LOGCR,LOGCM,M,MWAPP,MWRAPP,MZAPP
00104    26*            DOUBLE PRECISION SEB1,SIGMA
00104    27*      C
00104    28*      C *** READ THE NAME AND RUN NO. OF THE EXPERIMENT***
00104    29*      C
00105    30*           20 READ (1,1,END=21) TITLE
00110    31*              WRITE(3,200) TITLE
00113    32*      200  FORMAT(1H1/30X,20A4)
00114    33*         1 FORMAT (20A4)
00114    34*      C
00114    35*      C *** READ EXPERIMENTAL CONSTANTS***
00114    36*      C
00115    37*           22 READ (1,23) CO,M,T,OMEGA,REVC,SMIN,SEC,CF,VBAR,
00115    38*             * VSORCE                ,RHO, RSORCE                , BUFFER
00134    39*           23 FORMAT (3F10.0/5F10.0/F10.0,16A4/F10.0,16A4/20A4)
00134    40*      C
00134    41*      C *** CALCULATE ROTOR STRETCH AT THE ROTOR REFERENCE POSITION***
00134    42*      C
00135    43*              REFPSI = 5.200 + OMEGA*1.32E-07 + OMEGA*OMEGA*7.71E-12
00136    44*              REFPSO = 7.300 + OMEGA*1.63E-07 + OMEGA*OMEGA*6.32E-12
00137    45*              REFPOS = (REFPSI + REFPSO)/2.
00137    46*      C
00137    47*      C *** READ FRINGE INTERVAL,FRINGE WIDTH,WHITE LIGHT LIGHT OPTION
00137    48*      C *** (IHINGE GT 0),NO. OF FRINGES,NO. OF DUPLICATE READINGS,
00137    49*      C *** NO OF TRIES AT FITTING DATA
00137    50*      C
00140    51*              READ (1,15) CSPACE,FWIDTH,IHINGE,NFRNGE,NDUP,NTRY
00150    52*           15 FORMAT (2F10.0,4I3)
```

84294

```
00151      53*            N = NFRNGE + 2
00152      54*            N1 = NFRNGE + 1
00152      55*      C
00152      56*      C ***  READ BASE LINE CORRECTIONS***
00152      57*      C
00153      58*            READ (1,51) (DSPLC1(I),I = 2,N1)
00161      59*         51 FORMAT (8F10.0)
00162      60*            DSPLC1(1) = 0.0
00163      61*            DSPLC1(N) = 0.0
00163      62*      C
00163      63*      C ***  CALCULATE REVISED SPEED FROM TACHOMETER-STOPWATCH-DATA IF GIVEN
00163      64*      C
00164      65*            OMEGA1 = OMEGA
00165      66*            IF (SMIN.LE.0.) GO TO 99
00167      67*            IF (OMEGA.GE.12590.) GO TO 97
00171      68*            REVC = REVC*1000.
00172      69*            GO TO 98
00173      70*         97 REVC = REVC*6900.
00174      71*         98 TSEC = (SMIN*60. + SEC)*CF
00175      72*            OMEGA = REVC/TSEC*6.28318
00176      73*            OMEGA1 = OMEGA/0.104719755
00177      74*            GO TO 100
00200      75*         99 OMEGA = OMEGA*0.104719755
00201      76*        100 OMEGA = OMEGA*OMEGA
00202      77*            R1 = 8.31432E07
00202      78*      C
00202      79*      C ***  PRINT OUT EXPERIMENTAL CONSTANTS***
00202      80*      C
00203      81*            WRITE (3,84) CO,VBAR, VSORCE                ,RHO,
00203      82*           * RSORCE                ,OMEGA1,T,M, BUFFER
00214      83*      84    FORMAT(1H1,30X,21HINITIAL CONCENTRATION,F8.2,8H FRINGES//
00215      84*           *                30X,23HPARTIAL SPECIFIC VOLUME,F6.3,4H CC/GM,1AA4//
00215      85*           *                30X,15HSOLVENT DENSITY,7X,F8.3,6H GM/CC,16A4//
00216      86*           *                30X,5HSPEED,17X,F7.0,7H R.P.M.//
00216      87*           *                30X,20HABSOLUTE TEMPERATURE,F9.2//
00216      88*           *                30X,20HMAGNIFICATION FACTOR,F9.4//
00216      89*           *                30X,8HBUFFER, ,20A4)
00217      90*            A1 = 2.*R1*T/((1.-VBAR*RHO1*OMEGA)
00217      91*      C
00217      92*      C ***  READ POSITION OF THE INNER AND OUTER REFERENCES ON COMPARATOR***
00217      93*      C
00220      94*            READ (1,3) (RI(K),K = 1,NOUP)
00226      95*            READ (1,3) (RO(K),K = 1,NOUP)
00234      96*            RIM = XMEAN(RI,NOUP)
00235      97*            ROM = XMEAN(RO,NOUP)
00236      98*            REFP = (RIM + ROM)/2.
00236      99*      C
00236     100*      C ***  READ POSITION OF MENISCUS ON COMPARATOR***
00236     101*      C
00237     102*            READ (1 ,3) (RC(I,1),I = 1,NOUP)
00245     103*            RM = XMEAN(RC(1,1),NOUP)
00246     104*            XYZ = 1.0
00247     105*            IF (RIM.LE.RM)GO TO 91
00251     106*         90 XYZ = -1.0
00251     107*      C
00251     108*      C ***  FIND ACTUAL POSITION OF MENISCUS RELATIVE TO AXIS OF ROTATION***
00251     109*      C
00252     110*         91 R(I) = XYZ*(RM-REFP)*M/10. + REFPOS
00253     111*            DO 30 I = 2,N1
```

```
00253     112*      C
00253     113*      C *** READ POSITION OF EACH FRINGE AND CONVERT TO ACTUAL POSITION
00253     114*      C     RELATIVE TO THE AXIS OF ROTATION***
00253     115*      C
00256     116*            READ (1 ,3) (RC(I,L),I = 1,NDUP)
00269     117*            RCM(L) = XMEAN(RC(1,L),NDUP)
00265     118*            X=RCM(L)
00266     119*         30 R(L) = XYZ*(X-REFP)*M/10. + REFPOS
00266     120*      C
00266     121*      C *** READ POSITIONOF BASE AND CALCULATE ACTUAL POSITION RELATIVE TO
00266     122*      C     AXIS OF ROTATION***
00266     123*      C
00270     124*            READ (1 ,3) (RC(I,N),I = 1,NDUP)
00276     125*            RBM = XMEAN(RC(1,N1),NDUP)
00277     126*            R(N) = XYZ*(RBM-REFP)*M/10. + REFPOS
00300     127*          3 FORMAT (8F10.0)
00300     128*      C
00300     129*      C *** PRINT OUT ALL FRINGE DATA***
00300     130*      C
00301     131*            WRITE (3,200) TITLE
00304     132*            WRITE (3 ,35) (RI(I),RO(I),I = 1,NDUP)
00313     133*         35 FORMAT (//34X,15HINNER REFERENCE,32X,15HOUTER REFERENCE
00313     134*           //(36X,F8.3,40X,F8.3))
00314     135*            WRITE (3 ,36) RIM,ROM,REFP,REFPOS
00322     136*         36 FORMAT(//3AX,F8.3,40X,F8.3//31X,32HREFERENCE POSITION ON COMPARATOR
00322     137*           *,F8.3/29X,30HACTUAL REFERENCE POSITION (CM),F8.4)
00323     138*            WRITE (3 ,37) (RC(J,1),J = 1,NDUP)
00331     139*         37 FORMAT (/59X,8HMENISCUS//(59X,F8.3))
00332     140*            WRITE (3 ,38) RM
00335     141*         38 FORMAT (/59X,F8.3)
00336     142*            LIM1 = 0
00337     143*            LIM = (47-2*NDUP)/(7+NDUP)
00340     144*            L2 = 1
00341     145*         32 L1 = L2+1
00342     146*            L2 = L1+9
00343     147*            L3 = MIN0(L2,N1)
00344     148*            WRITE (3 ,43)
00346     149*            DO 44 I = 1,NDUP
00351     150*         44 WRITE (3 ,31) (RC(I,J)          ,J = L1,L3)
00360     151*         31 FORMAT (5X,10(1F8.3,4X))
00361     152*            WRITE (3 ,43)
00363     153*         43 FORMAT (/)
00364     154*            WRITE (3 ,31) (RCM(J)          ,J = L1,L3)
00372     155*            LIM1 = LIM1 + 1
00373     156*            LIM = 60/(NDUP+7)
00374     157*            LIM1 = 0
00375     158*         53 IF (L2.NE.N1.AND. .L2.LE.L3)GO TO 33
00377     159*            WRITE (3 ,41) (RC(J,N),J = 1,NDUP)
00405     160*         41 FORMAT (/41X,6HBOTTOM//(59X,F8.3))
00406     161*            WRITE (3 ,42) RBM
00411     162*         42 FORMAT (/59X,F8.3)
00411     163*      C
00411     164*      C *** CONVERT DISPLACEMENTS (IN CM) TO FRINGE UNITS AND FORM VECTOR OF R-SQUARED
00411     165*      C
00412     166*            DO 8 I = 1,N
00415     167*            DSPL C1(I) = DSPL C1(I)/F-IDTH
00416     168*          8 RSQ(I) = R(I)*R(I)
00420     169*            DO 92 IPLAS = 1,NTRY
00423     170*            WRITE (3,200) TITLE
```

84296

```
00426   171*            WRITE (3,204)
00430   172*       204  FORMAT (/50H FRINGES LABELLED BY CONSERVATION OF MASS ARGUMENT/)
00431   173*            WRITE (3,201)
00433   174*       201  FORMAT (/23H REGRESSION INFORMATION//16H VALUES OF CO-CM/)
00433   175*     C
00433   176*     C *** READ REGRESSION SCHEME***
00433   177*     C
00434   178*            READ (1 ,93) NREG,(NR(I),NP(I),I = 1,NREG),NILAB
00445   179*            NLAB=1
00446   180*            NHINGE=0
00447   181*            IF(IHINGE.GT.0.0)NHINGE=1
00451   182*        93  FORMAT (2A12)
00451   183*     C
00451   184*     C *** CALCULATE THE INTEGRAL FOR CONSERVATION OF MASS***
00451   185*     C
00452   186*            CIMCM = (R(2)-R(1))/(R(3)-R(2))*CSPACE
00453   187*            CRMCN = (R(N)-R(N1))/(R(N1)-R(NFRNGE ))*CSPACE
00454   188*            CONMAS  = RSQ(2)/2. + RSQ(N1)/2.
00455   189*            DO 4 I = 3,NFRNGE
00460   190*          4 CONMAS  = CONMAS  + RSQ(I)
00462   191*            CONMAS  = CONMAS *CSPACE
00463   192*            CONCM1 = -1500.
00463   193*     C
00463   194*     C *** ITERATE UNTIL VALUE FOR INTEGRAL HAS CONVERGED***
00463   195*     C
00464   196*            DO 7 J = 1,30
00467   197*            CRMCM = CIMCM + FLOAT(N1-2)*CSPACE + CRMCN
00470   198*            CNMASS = CONMAS  + CIMCM*(RSQ(2)+RSQ(1))/2.
00471   199*            CNMASS = CNMASS + CRMCN*(RSQ(N)+RSQ(N1))/2.
00472   200*            CONCN = RSQ(N)*CRMCM - CNMASS
00473   201*            CONCM = CONCM/(RSQ(N) - RSQ(1))
00474   202*            WRITE(3,900)CONCM
00477   203*       900  FORMAT (1X,F12.5)
00500   204*            IF (ABS(CONCM-CONCM1).LT.1.0E-04)GO TO 951
00502   205*       24   C(1) = CO - CONCM
00503   206*            C(2) = C(1) + CIMCM
00504   207*            DO 5 I = 3,N1
00507   208*          5 C(I) = C(I-1) + CSPACE
00511   209*            DO 50 I = 2,N1
00514   210*            DSPL CE(I) = DSPL CI(I)
00515   211*            C(I) = C(I) + DSPL CE(I)
00516   212*       50   LOGC(I) = ALOG(C(I))
00520   213*            DSPL CE(1) = 0.0
00521   214*            DSPL CE(N) = 0.0
00522   215*            LIM = 2
00523   216*            DO 68 I = 1,NREG
00523   217*     C
00523   218*     C *** REGRESS LOG CONCENTRATION ON RADIUS-SQUARED***
00523   219*     C
00524   220*            CALL POLREG(RSQ(LIM),LOGC(LIM),ELOGC(LIM),NR(I),NP(I),2,A1(1,I),
00526   221*            SER(1,I),SIGMA,0)
00527   222*       68   LIM = LIM + NR(I)
00531   223*            NN = NP(1) + 1
00532   224*            LOGCM = A1(1,1)
00533   225*            DO 6 I = 2,NN
00536   226*          6 LOGCM = LOGCM + A1(I,1)*RSQ(1)**(I-1)
00540   227*            NN = NP(NREG) + 1
00541   228*            LOGCB = A1(1,NREG)
00542   229*            DO 81 I = 2,NN
```

```
00545   230*          P1 LOGCP = LOGCB + B1(I,NREG)*RSQ(N)**(I-1)
00547   231*             LOGC(1) = LOGCM
00550   232*             LOGC(N) = LOGCB
00551   233*             FLOGC(1) = LOGCM
00552   234*             FLOGC(N) = LOGCB
00553   235*             C(1) = EXP(LOGCM)
00554   236*             C(N) = EXP(LOGCB)
00555   237*             CINCP = C(2) - C(1)
00556   238*             CBMCB = C(N) - C(N-1)
00557   239*          3  CONCM1 = CONCM
00561   240*             WRITE (3 ,29) J
00564   241*          29 FORMAT (53H CONSERVATION OF MASS TREATMENT NOT CONVERGENT AFTER ,
00564   242*            *I3,11H ITERATIONS )
00564   243*       C
00564   244*       C *** PRINT OUT REGRESSION CO-EFFICIENTS AND STANDARD ERRORS***
00564   245*       C
00565   246*       91    WRITE (3 ,57)
00567   247*          57 FORMAT (/21H REGRESSION SCHEME...)
00570   248*             DO 2 I = 1,NREG
00573   249*              WRITE (3 ,43)
00575   250*          43 NN = NP(I) + 1
00576   251*             WRITE (3 ,54) NR(I),NP(I)
00602   252*          54 FORMAT (1HO,1X,I3,15H POINTS, DEGREE,I2,11H POLYNOMIAL)
00603   253*             WRITE (3 ,55) (B1(J,I),J = 1,NN)
00611   254*          55 FORMAT (/35X,13HCOEFFICIENTS:,5X,8F12.5)
00612   255*             WRITE (3,211)
00614   256*          211 FORMAT (/1X,31HVARIANCE-COVARIANCE MULTIPLIER:)
00615   257*             WRITE (3,210) SES1(1,1)
00620   258*             DO 209 KK1=2,NN
00623   259*             SS(1)=SES1(KK1,1)
00624   260*             MM=NN-1
00625   261*             LL1=KK1
00626   262*             DO 208 KK2=2,KK1
00631   263*             LL1=LL1+MM
00632   264*             SS(KK2)=SES1(LL1,1)
00633   265*          208 MM=MM-1
00635   266*          209 WRITE (3,210) (SS(NN1),NN1=1,KK1)
00644   267*          210 FORMAT (5(6X,6E12.5))
00645   268*              WRITE (3 ,56) SIGMA
00651   269*           56 FORMAT (/42(),8HSIGMA:,8X,F13.5)
00652   270*              DO 70 I = 1,N
00655   271*           70 DEV(I) = LOGC(I) - ELOG(I)
00655   272*       C
00655   273*       C *** CALCULATE THE APPARENT WEIGHT-AVERAGE MOLECULAR WEIGHT***
00655   274*       C
00657   275*              MWAPP = A1*CONCM/(CO*(RSQ(N)-RSQ(1)))
00660   276*              LIM = 1
00661   277*              NR(I) = NR(I) + 1
00662   278*              NR(NREG) = NR(NREG) + 1
00663   279*              DO 67 I = 1,NREG
00666   280*              LIM1 = LIM + NP(I) - 1
00667   281*              NPX = NP(NREG)
00670   282*              DO 75 L = LIM,LIM1
00673   283*              APP = B1(2,I)
00674   284*              IF (NPX.EQ.1) GO TO 75
00674   285*       C
00674   286*       C *** CALCULATE THE SLOPE OF THE REGRESSION LINE AND HENCE THE
00674   287*       C     APPARENT MOLECULAR WEIGHT AT EACH OBSERVED POINT***
00674   288*       C
```

84298

```
00676          DO 71 KK = 2,NPX
00701       71 APP = APP + FLOAT(KK)*BI(KK+1,I)*RSQ(L)**(KK-1)
00703       75 MWRAPP(L) = APP*AI
00705       69 LIM = LIM1 + 1
00707          NR(1) = NR(1) - 1
00710          NR(NREG) = NR(NREG) - 1
00711          MZAPP = (MWRAPP(N)*C(N) - MWRAPP(1)*C(1))/CBMCM
00711    C
00711    C *** PRINT RESULTS
00711    C
00712          WRITE (3,200) TITLE
00715          WRITE (3,9)                   (R(I),C(I),DSPLCE(I),LOGC(I),
00715          RSQ(I),ELOGC(I),DEV(I), MWRAPP(I),I = 1,N)
00732        9 FORMAT (/11X,3HAPP/    8H RADIUS,6X,13HCONCENTRATION,5X,
00732         *9HBASE-LINE,7X,5HLOG C,7X,9HR-SQUARED,8X,6HFITTED,7X,10HDEVIATIONS
00732         *,9X,1HM/      15X,12H(IN FRINGES),4X,11HCORRECTIONS,34X,5HLOG C,
00732         *27X,3HC,R//(1X,F7.4,8X,F7.2,8X,F7.2,8X,F7.4,6X,F9.4,8X,F7.4,8X,
00732         *F7.4,8X,F7.3))
00733          NU = 53 - NREG
00734          NT = N + NREG + 2
00735       63 WRITE (3,16) MWAPP,MWRAPP(1),MWRAPP(N),MZAPP
00743       16 FORMAT (3(/),11X,3HAPP,/10X,1HM,3X,F10.0/11X,1HW
00743         *             //11X,3HAPP/10X,1HM,3X,F10.0/11X,3HW,M
00743         *             //11X,3HAPP/10X,1HM,3X,F10.0/11X,3HW,B
00743         *             //11X,3HAPP/10X,1HM,3X,F10.0/11X,1HZ )
00743  915     IF(NHINGE.LE.0)GO TO 914
00744    C
00744    C *** IF WHITE LIGHT HINGE POSITION IS GIVEN PERFORM FRINGE LABELLING***
00744    C
00746          WRITE (3,200) TITLE
00751          WRITE (3,205)
00753      205 FORMAT (/39H FRINGE LABELLING BY HINGE-POINT METHOD/)
00755          WRITE (3,206)
00756      206 FORMAT (/23H REGRESSION INFORMATION//)
00757       11 DO 12 I = 2,N
00762          C(I) = C0 - FLOAT(IHINGE-I)*CSPACE
00763          C(I) = C(I) + DSPLCE(I)
00765       12 LOGC(I) = ALOG(C(I))
00766  960     LIM = 2
00767          DO 82 I = 1,NREG
00767    C
00767    C *** REGRESS LOG CONCENTRATION ON RADIUS SQUARED***
00767    C
00772          CALL POLREG (RSQ(LIM),LOGC(LIM),ELOGC(LIM),NR(I),NP(I),2,BI(1,I),
00772         *SEBI(1,I),SIGMA,0)
00773       82 LIM = LIM + NR(I)
00775          NN = NP(1) + 1
00776          LOGCM = BI(1,1)
00777          DO 66 I = 2,NN
01002       66 LOGCM = LOGCM + BI(I,1)*RSQ(1)**(I-1)
01004          NN = NP(NREG) + 1
01005          LOGCB = BI(1,NREG)
01006          DO 80 I = 2,NN
01011       80 LOGCB = LOGCB + BI(I,NREG)*RSQ(N)**(I-1)
01013          LOGC(1) = LOGCM
01014          LOGC(N) = LOGCB
01015          C(1) = EXP(LOGCM)
01016          C(N) = EXP(LOGCB)
01017          ELOGC(1) = LOGCM
```

```
01020     348*            ELOGC(N) = LOGCB
01021     349*            NHINGE=0
01022     350*            GO TO 951
01023     351*      9;0   IF(NLAB.EQ.0)GO TO 92
01023     352*      C
01023     353*      C *** RELABEL FRINGES SO THAT THE FRINGE NEAREST THE INITIAL
01023     354*      C     CONCENTRATION IS LABELLED WITH EXACTLY THAT CONCENTRATION
01023     355*      C
01025     356*            WRITE (3,200) TITLE
01030     357*            WRITE (3,207)
01032     358*        207 FORMAT(/124H FRINGES RELABELLED SUCH THAT THE FRINGE LOCATED CLOSE
01032     359*           ST TO THE INITIAL CONCENTRATION IS ASSIGNED EXACTLY THAT CONCENTRA
01032     360*          *TION/)
01033     361*            WRITE (3,206)
01035     362*            N11=IFIX(1.0/CSPACE)
01036     363*            N12=N11AB+2
01037     364*            DO 901 I=N12,N1,N11
01042     365*            CF=C(I)-CO
01043     366*            IF(ABS(CF).LT.0.5)GOTO 902
01045     367*      901   CONTINUE
01047     368*      902   DO 903 I=1,*
01052     369*            C(I)=C(I)=CF
01053     370*      903   ELOGC(I)=ALOG (C(I))
01055     371*            NLAB=0
01056     372*            GO TO 950
01057     373*         92 CONTINUE
01061     374*            GO TO 20
01062     375*        -21 WRITE (3,202)
01064     376*        202 FORMAT (1H1/)
01065     377*            STOP
01066     378*            END
```

```
00101       1*            SUBROUTINE POLREG (X,Y1,FY,N,NP,IOP,BETA,SS,SIGMA,IPRNT)
00101       2*      C     ********************************************************************
00101       3*      C     TO CARRY OUT AN ORTHOGONAL POLYNOMIAL REGRESSION OF COLUMN VECTOR
00101       4*      C     Y ON COLUMN VECTOR T
00101       5*      C     LENGTH OF REGRESSION VECTORS = N
00101       6*      C     DEGREE OF POLYNOMIAL FITTED = NP.
00101       7*      C     IF IOP = 1 A0 CONSTANT TERM IS INCLUDED IN THE REGRESSION
00101       8*      C     IF IOP = 2 A CONSTANT TERM IS INCLUDED IN THE REGRESSION
00101       9*      C     REGRESSION COEFFICIENTS ARE CALCULATED IN COLUMN VECTOR BETA
00101      10*      C     ORTHOGONAL REGESSION COEFFICIENTS ARE CALCULATED IN COLUMN VECTOR
00101      11*      C     GAMMA
00101      12*      C     VARIANCE-COVARIANCE MULTIPLIER IS CONTAINED IN SS
00101      13*      C     SIGMA RETURNS THE SQUARE ROOT OF THE RESIDUAL SUM OF SQUARES
00101      14*      C     IF IPRNT IS POSITIVE GET ANALYSIS OF VARIANCE TABLE
00101      15*      C     IF IPRNT IS 0 OR LESS NO ANALYSIS OF VARIANCE TABLE IS PRINTED
00101      16*      C     ********************************************************************
00101      17*      C
00103      18*            DIMENSION T(100), Y(100), BETA(1), SS(1),Z(100,6), A(100,6),
00103      19*           *FY(1)
00104      20*            DIMENSION TOTZ(50), GAMMA(50), X(1), Y1(1)
```

```
      DOUBLE PRECISION T,TT,SS,Z,A,DOTZ,GAMMA,ZDOTY,C,D,TSS,RSS,RMS,SIGM
     1A,Y,CONST,SB,BTA
      DOUBLE PRECISION DOT
      DO 1 I=1,N
      Y(I)=Y(I)
      T(I)=X(I)
      NN=NP+IOP-1
      A(1,1)=1.0
      A(2,1)=0.0
      A(2,2)=1.0
      IF (IOP-1) 3,2,4
    2 TT=DOT(T,T,-N)/DOT(T,T,N)
      DO 3 I=1,N
    3 Z(I,1)=T(I)
      DO 7 I=2,NP
      TT=0.0
      DO 5 I=1,N
    5 TT=TT+T(I)
      TT=TT/FLOAT(N)
      DO 6 I=1,N
    6 Z(I,1)=T(I)-TT
    4 Z(I,2)=T(I)-TT
    7 S(1,2)=-TT
      I1=NP-1
      DOTZ(1)=DOT(Z(1,1),Z(1,1),N)
      DO 8 J=2,NP
      DOTZ(J)=DOT(Z(1,J),Z(1,J),N)
      C=DOTZ(1,1)/DOTZ(1,2)
      D=DOTZ(1,1)/DOTZ(J-1)
      JI=J+1
      J2=J-1
      CALL POLRAS (Z(1,1),Z(1,J),Z(1,J2),Z(
      CONST=-TT*C
      A(J,J)=1.0
      A(J,J1)=CONST+A(J2,J)
      A(1,J1)=CONST*A(1,1)-D*A(1,J2)
      DO 9 K=2,J2
      A(K,J1)=A(K-1,J1)+CONST*A(K,J1)-D*A(K,J2
      DOTZ(K)=DOT(Z(1,K),Z(1,N),N)
    9 J=1,N1
      L=J+1
      DO 9 I=L,N
      S(I,J1)=0.0
      TSS=DOTZ(1,1)
      DSS=TSS
      DO 10 I=1,N
      ZDOTY=DOT(Z(1,1),Y,N)
      GAMMA(1)=ZDOTY/DOTZ(1)
      SSQ(1)=GAMMA(1)*ZDOTY
      DSS=DSS-SSQ(1)
   10 DIF=FLOAT(N-IOP+1)
      DIF=FLOAT(N-IOP-1)
      IF (IOP*IOP-2) 21, 20, 11
      TSS=TSS-SSQ(1)
   11 DSS=DSS/DIF
      SIGM=SQRT(DSS)
      RSS=DSS*DIF
      IF (IOP-NP) 14,14,12
```

```
00237    80*      12    WRITE (3,19)
00241    81*            M1=MM-1
00242    82*            DO 13 I=1OP,M1
00245    83*            IDEGRF=I-1OP+1
00246    84*      13    WRITE (3,20) IDEGRE,SS(I)
00253    85*            IDEGRE=MM-1OP+1
00254    86*            WRITE (3,21) IDEGRE,SS(MM),F
00261    87*            IRDF=IFIX(RDF)
00262    88*            WRITE (3,22) IRDF,RSS,RMS
00267    89*            ITDF=IFIX(TDF)
00277    90*            WRITE (3,23) ITDF,TSS
00274    91*      14    DO 16 I=1,M
00277    92*            E=0.0
00300    93*            DO 15 J=1,MM
00303    94*      15    E=E+Z(I,J)*GAMMA(J)
00305    95*      16    EY(I)=E
00307    96*            M=0
00310    97*            DO 18 I=1,MM
00313    98*            BTA=0.0
00314    99*            DO 17 J=1,MM
00317   100*            N=M+1
00320   101*            SB=0.0
00321   102*            DO 24 K = J,MM
00324   103*        24  SB=SB+ A(I,K)*A(J,K)/DOT7(K)
00326   104*            SS(N)=SB
00327   105*      17    BTA=BTA+A(I,J)*GAMMA(J)
00331   106*      18    BETA(I)=BTA
00333   107*            MP=MM-1OP+1
00334   108*            RETURN
00334   109*      C
00335   110*      19    FORMAT (/49X,45H ANALYSIS OF VARIANCE, POLYNOMIAL REGRESSION /45X,
00335   111*           1494(1H-)//30X,19HSOURCE OF VARIATION,12X,4HDF,13X,4HSS,10X,4HMS,
00335   112*           2F,10X,1HF/25X,84(1H-)/)
00336   113*      20    FORMAT (26X,6HDEGREE,I3,28X,1H1,9X,D16.9)
00337   114*      21    FORMAT (26X,6HDEGREE,I3,29X,1H1,9X,D16.9,21X,F8.3,6H (P =,F7.4,1H)
00337   115*           1)
00340   116*      22    FORMAT (26X,9HRESIDUALS,25X,I9,9X,D16.9,5X,D16.9,5X,F8.3)
00341   117*      23    FORMAT (25X,84(1H-)/26X,5HTOTAL,29X,I9,9X,D16.9/25X,84(1H-)/)
00342   118*            END
```

```
00100     1*      C     TO DETERMINE THE DOT PRODUCT OF COLUMN VECTORS X AND Y***
00101     2*            DOUBLE PRECISION FUNCTION DOT (X,Y,N)
00103     3*            DOUBLE PRECISION X,Y
00104     4*            DIMENSION X(1), Y(1)
00105     5*            IF (N.LT.0) GO TO 2
00107     6*            DOT=0.0
00110     7*            DO 1 I=1,N
00113     8*      1     DOT=DOT+X(I)*Y(I)
00115     9*            GO TO 4
00116    10*      2     N=-N
00117    11*            DOT=0.0
00120    12*            DO 3 I=1,N
00123    13*      3     DOT=DOT+X(I)*X(I)*Y(I)
00124    14*      4     RETURN
00126    15*            END
```

```
00101     1*            SUBROUTINE POLBAS (Z2,Z1,Z0,Z0P,C,D,N)
00101     2*      C *** TO CONSTUCT ORTHOGONALISED VARIATES FROM POLYREG***
00103     3*            DOUBLE PRECISION Z2,Z1,Z0,Z0P,C,D
00104     4*            DIMENSION Z0(1), Z1(1), Z2(1), Z0P(1)
00105     5*            DO 1 I=1,N
00110     6*      1     Z2(I)=(Z0P(I)-C)*Z1(I)-D*Z0(I)
00112     7*            RETURN
00113     8*            END
```

```
00101     1*            FUNCTION XMEAN(X,N)
00101     2*      C *** TO CALCULATE THE MEAN OF N VARIBLES IN COLUMN VECTOR X***
00103     3*            DIMENSION X(1)
00104     4*            XMEAN = 0.0
00105     5*            DO 1 I=1,N
00110     6*      1     XMEAN=XMEAN+X(I)
00112     7*            XMEAN=XMEAN/FLOAT(N)
00113     8*            RETURN
00114     9*            END
```

RUN 1371 MET-HAEMOGLOBIN A PH 5.4, I = 0.25 WITH 5 MOLES OF ATP PER TETRAMER

INITIAL CONCENTRATION 19.69 FRINGES
PARTIAL SPECIFIC VOLUME .746 CC/GM
SOLVENT DENSITY 1.008 GM/CC
SPEED 9000. R.P.M.
ABSOLUTE TEMPERATURE 293.16
MAGNIFICATION FACTOR .4562
BUFFER, PH 5.4 IONIC STRENGTH .25 (SODIUM ACETATE) 20 DEGREES

84310

RUN 1341 MET-HAEMOGLOBIN A PH 5.4, I = 0.25 WITH 5 MOLES OF ATP PER TETRAMER

INNER REFERENCE	OUTER REFERENCE
7.262	42.222
7.261	42.224
7.263	42.230
7.259	42.225
7.264	42.227
7.262	42.226

REFERENCE POSITION ON COMPARATOR 29.744

ACTUAL REFERENCE POSITION (CM) 6.5019

MENISCUS

31.719									
31.719									
31.723									
31.720									
31.717									
31.720									
31.794	32.371	32.915	33.430	33.926	34.378	34.766	35.132	35.517	35.842
31.792	32.361	32.924	33.429	33.920	34.367	34.762	35.128	35.522	35.851
31.788	32.367	32.925	33.431	33.924	34.367	34.767	35.133	35.522	35.850
31.788	32.370	32.920	33.436	33.929	34.370	34.763	35.133	35.520	35.851
31.790	32.365	32.919	33.444	33.928	34.367	34.770	35.137	35.521	35.849
31.790	32.367	32.921	33.434	33.925	34.370	34.766	35.133	35.520	35.849
36.182	36.500	36.763	37.049	37.299	37.546	37.770	38.012	38.226	
36.186	36.503	36.754	37.046	37.300	37.543	37.782	38.007	38.230	
36.182	36.494	36.759	37.053	37.301	37.546	37.771	38.002	38.234	
36.180	36.495	36.758	37.056	37.298	37.545	37.786	38.010	38.240	
36.183	36.503	36.759	37.054	37.298	37.550	37.785	37.995	38.225	
36.183	36.499	36.759	37.052	37.299	37.546	37.779	38.005	38.231	

BOTTOM

38.438
38.435
38.436
38.432
38.440
38.436

RUN 1391 MET-HAEMOGLOBIN A PH 5.9, I = 0.25 WITH 5 MOLES OF ATP PER TETRAMER

FRINGES LABELLED BY CONSERVATION OF MASS ARGUMENT

REGRESSION INFORMATION

VALUES OF CO-CM

.78618+01
.78065+01
.78065+01

REGRESSION SCHEME...

19 POINTS, DEGREE 2 POLYNOMIAL

COEFFICIENTS: .64529 -.12039 .00363

VARIANCE-COVARIANCE MULTIPLIER:

.15638+06
-.64246+04 .26398+03
.65943+02 -.27100+01 .27823-01

SIGMA: .19798-002

84316

RUN 1341 MET-HAEMOGLOBIN A PH 5.4, I = 0.25 WITH 5 MOLES OF ATP PER TETRAMER

RADIUS	CONCENTRATION (IN FRINGES)	BASE-LINE CORRECTIONS	LOG C	R-SQUARED	FITTED LOG C	DEVIATIONS	APP M A.R
6.8201	11.88	.00	2.4751	46.5143	2.4751	.0000	46081.
6.8234	11.95	.00	2.4807	46.5583	2.4843	-.0036	46152.
6.8497	12.95	.00	2.5611	46.9179	2.5597	.0014	46730.
6.8749	13.95	.00	2.6355	47.2646	2.6333	.0022	47287.
6.8983	14.95	.00	2.7047	47.5872	2.7026	.0021	47805.
6.9208	15.95	.00	2.7695	47.8970	2.7698	-.0003	48303.
6.9410	16.95	.00	2.8303	48.1780	2.8314	-.0011	48759.
6.9591	17.95	.00	2.8876	48.4290	2.8869	.0007	49158.
6.9758	18.95	.00	2.9418	48.6623	2.9388	.0030	49532.
6.9935	19.95	.00	2.9932	48.9094	2.9943	-.0011	49930.
7.0085	20.95	.00	3.0421	49.1191	3.0418	.0004	50266.
7.0237	21.95	.00	3.0889	49.3329	3.0905	-.0017	50610.
7.0382	22.95	.00	3.1333	49.5359	3.1370	-.0037	50936.
7.0500	23.95	.00	3.1760	49.7027	3.1755	.0005	51204.
7.0634	24.95	.00	3.2169	49.8914	3.2192	-.0023	51507.
7.0747	25.95	.00	3.2562	50.0511	3.2564	-.0003	51764.
7.0859	26.95	.00	3.2940	50.2105	3.2938	.0002	52020.
7.0966	27.95	.00	3.3304	50.3611	3.3293	.0012	52262.
7.1069	28.95	.00	3.3656	50.5078	3.3640	.0016	52498.
7.1172	29.95	.00	3.3995	50.6543	3.3988	.0008	52733.
7.1265	30.89	.00	3.4306	50.7877	3.4306	.0000	52947.

APP
A 50037.

APP
M 46081.
.M

APP
R 52947.
.R

APP
Z 57234.

RUN 1341 MET-HAEMOGLOBIN A PH 5.4, I = 0.25 WITH 5 MOLES OF ATP PER TETRAMER

FRINGES RELABELLED SUCH THAT THE FRINGE LOCATED CLOSEST TO THE INITIAL CONCENTRATION IS ASSIGNED EXACTLY THAT CONCENTRATION

REGRESSION INFORMATION

REGRESSION SCHEME...

17 POINTS, DEGREE 2 POLYNOMIAL

COEFFICIENTS: -.25345 -.09419 .00332

VARIANCE-COVARIANCE MULTIPLIER:

.15638+06
-.64296+04 .26398+03
.65993+02 -.27100+01 .27823-01

SIGMA: .20264-002

84318

RUN 1341 MET-HAEMOGLOBIN A PH 5.4, I = 0.25 WITH 5 MOLES OF ATP PER TETRAMER

RADIUS	CONCENTRATION (IN FRINGES)	BASE-LINE CORRECTIONS	LOG C	R-SQUARED	FITTED LOG C	DEVIATIONS	APP M W.R
6.8201	11.62	.00	2.4531	46.5143	2.4531	.0000	47076.
6.8234	11.69	.00	2.4587	46.5583	2.4624	-.0037	47141.
6.8497	12.69	.00	2.5408	46.9179	2.5394	.0014	47670.
6.8749	13.69	.00	2.6167	47.2646	2.6144	.0022	48179.
6.8983	14.69	.00	2.6872	47.5872	2.6849	.0022	48653.
6.9208	15.69	.00	2.7530	47.8970	2.7533	-.0003	49109.
6.9410	16.69	.00	2.8148	48.1780	2.8159	-.0011	49522.
6.9591	17.69	.00	2.8730	48.4290	2.8722	.0008	49891.
6.9758	18.69	.00	2.9280	48.6623	2.9250	.0030	50234.
6.9935	19.69	.00	2.9801	48.9094	2.9812	-.0011	50597.
7.0085	20.69	.00	3.0297	49.1191	3.0293	.0004	50905.
7.0237	21.69	.00	3.0769	49.3329	3.0786	-.0017	51220.
7.0382	22.69	.00	3.1219	49.5359	3.1257	-.0037	51518.
7.0500	23.69	.00	3.1651	49.7027	3.1646	.0005	51763.
7.0634	24.69	.00	3.2064	49.8914	3.2088	-.0024	52041.
7.0747	25.69	.00	3.2461	50.0511	3.2464	-.0003	52276.
7.0859	26.69	.00	3.2843	50.2105	3.2841	.0002	52510.
7.0964	27.69	.00	3.3211	50.3611	3.3199	.0012	52731.
7.1069	28.69	.00	3.3565	50.5078	3.3549	.0017	52947.
7.1172	29.69	.00	3.3908	50.6543	3.3900	.0008	53162.
7.1265	30.63	.00	3.4221	50.7877	3.4221	.0000	53358.

APP M Y 50037.

APP M Y,M 47076.

APP M Y,R 53358.

APP M Z 57192.

BIBLIOGRAPHY

Ackers, G.K. (1967) *J. Biol. Chem. 242*, 3026.

Adams, E.T., and Fujita, H. (1963) *"Ultracentrifugal Analysis in Theory and Experiment", J. W. Williams, Ed., Academic Press, New York.*

Adams, E.T. (1964) *Proc. Nat. Acad. Sci. U.S. 51*, 509.

Adams, E.T. (1965) *Biochemistry 4*, 1646.

Adams, E.T. (1967) *Fractions No. 3. Spinco Division of Beckman Instruments, Inc., Palo Alto, Calif.*

Adams, E.T., and Lewis, M.S. (1968) *Biochemistry 7*, 1044.

Ames, B.N., and Dubin, D.T. (1960) *J. Biol. Chem. 235*, 769.

Archibald, W.J. (1947) *J. Phys. Colloid Chem. 51*, 1204.

Armstrong, J. McD., and McKenzie, H.A. (1967) *Biochim. Biophys. Acta 147*, 93.

Armstrong, J. McD., McKenzie, H.A., and Sawyer, W.H. (1967) *Biochim. Biophys. Acta 147*, 60.

Arnone, A. (1972) *Nature 237*, 146.

Aschaffenburg, R., and Drewry, J. (1957) *Biochem. J. 65*, 273.

Ashman, L.K., and Atwell, J.L. (1972) *Biochim. Biophys. Acta 258*, 618.

Aune, K.C., Goldsmith, L.C. and Timasheff, S.N. (1971) *Biochemistry 10*, 1617.

Aune, K.C., and Timasheff, S.N. (1971) *Biochemistry 10*, 1609.

Babul, J., and Stellwagen, E. (1969) *Anal. Biochem. 28*, 216.

Baghurst, P.A. (1969), *Honours Thesis, Waite Agricultural Research Institute, University of Adelaide.*

Baldassare, J.J., Charache, S., Jones, R.T., and Ho, C. (1970) *Biochemistry 9*, 4707.

Bell, K., and McKenzie, H.A. (1964) *Nature 204*, 1275.

Bell, K., McKenzie, H.A., and Murphy, W.H. (1966) *Aust. J. Sci. 29*, 87.

Bell, K., McKenzie, H.A., and Shaw, D.C. (1966) *Aust. J. Sci. 29*, 86.

Bell, K., and McKenzie, H.A. (1967) *Biochim. Biophys. Acta 147*, 123.

Bell, R.P. (1959) *"The Proton in Chemistry", Methuen, London, p.187.*

Benesch, R., Macduff, G., and Benesch, R.E. (1965) *Anal. Biochem. 11*, 81.

Benesch, R., and Benesch, R.E. (1967) *Biochem. Biophys. Res. Commun. 26*, 162.

Benesch, R., Benesch, R.E., and Yu, C.I. (1968) *Proc. Nat. Acad. Sci. U.S. 59*, 526.

Benesch, R., and Benesch, R.E. (1969) *Nature 221*, 618.

Benesch, R.E., Benesch, R., and Macduff, G. (1964) *Biochemistry 3*, 1132.

Benesch R.E., Benesch, R., Renthal, R., and Gratzer, W.B. (1971) *Nature New Biology 234*, 174.

Bernfeld, P., Berkeley, B.J. and Bieber, R.E. (1965) *Arch. Biochem. Biophys. 111*, 31.

Berns, D.S. (1971) in *"Subunits In Biological Systems", Part A, Timasheff, S.N., and Fasman, G.D., Eds., Marcel Dekker, Inc., New York.*

Blake, A., and Peacocke, A.R. (1968) *Biopolymers 6*, 1225.

Brewer, G.J., and Eaton, J.W. (1971) *Science 171*, 1205.

Briehl, R.W. (1964) In *"Structure and Activity of Enzymes". T.W. Goodwin, J.I. Harris, and B.S. Hartley, Eds., Academic Press, London and New York, p.171.*

Bro, P., Singer, S.J., and Sturtevant, J.M. (1955) *J. Amer. Chem. Soc., 77*, 4924.

Brumbaugh, E.E., and Ackers, G.K. (1968) *J. Biol. Chem. 243*, 6315.

Bucci, E., and Fronticelli, C. (1965) *J. Biol. Chem. 240*, PC551.

Bucci, E., Fronticelli, C., Chiancone, E., Wyman, J., Antonini, E., and Rossi-Fanelli, A. (1965) *J. Mol. Biol. 12*, 183.

Cann, J.R. (1970) *"Interacting macromolecules: the theory and practice of their electrophoresis, ultracentrifugation and chromatography" Academic Press, New York.*

Casassa, E.F., and Eisenberg, H. (1964) *Adv. Protein Chem. 19*, 287.

Changeux, J.P., and Rubin, M.M. (1968) *Biochemistry 7*, 553.

Chanutin, A., and Curnish, R.R. (1967) *Arch. Biochem. Biophys. 121*, 96.

Chanutin, A., and Hermann, E. (1969) *Arch. Biochem. Biophys. 131*, 180.

Chen, P.S., Toribara, T.Y., and Warner, H. (1956) *Anal. Chem. 28*, 1756.

Chen, T.H., and Yang, J.T. (1971) *Biochem. Biophys. Res. Commun. 44*, 1285.

Chiancone, E., and Gilbert, G.A. (1965) *J. Biol. Chem. 240*, 3866.

Chiancone, E., Gilbert, L.M., Gilbert, G.A., and Kellett, G.L. (1968) *J. Biol. Chem. 243*, 1212.

Chun, P.W., and Kim, S.J. (1970) *J. Phys. Chem. 74*, 899.

Cooper, P.F., and Wood, G.C. (1968) *J. Pharm. Pharmacol. 20*, 150S.

Cox, D.J. (1967) *Arch. Biochem. Biophys. 119*, 230.

Cox, D.J. (1969) *Arch. Biochem. Biophys. 129*, 106.

Creeth, J.M., and Nichol, L.W. (1960) *Biochem. J. 77*, 230.

Creeth, J.M., and Pain, R.H. (1967) *Prog. Biophys. & Mol. Biol. 17*, 217.

Dalziel, K. (1968) *FEBS Lett. 1*, 346.

DeRenzo, E.C., Ioppolo, C., Amiconi, G., Antonini, E., and Wyman, J. (1967) *J. Biol. Chem. 242*, 4850.

DeVault, D. (1943) *J. Amer. Chem. Soc. 65*, 532.

Douglas, C.G., Haldane, J.S., and Haldane, J.B.S. (1912) *J. Physiol. 44*, 275.

Edelstein, S.J., and Schachman, H.K. (1967) *J. Biol. Chem. 242*, 366.

Edmond, E., Farquar, S., Dunstone, J.R., and Ogston, A.G. (1968) *Biochem. J. 108*, 755.

Fasman, G.D. (1963) in *"Methods in Enzymology" VI*, 928.

Field, E.O., and Ogston, A.G. (1955) *Biochem. J. 60*, 661.

Field, E.O., and O'Brien, J.R.P. (1955) *Biochem. J. 60*, 656.

Frieden, C. (1967) *J. Biol. Chem. 242*, 4045.

Frieden, C., and Colman, R.F. (1967) *J. Biol. Chem. 242*, 1705.

Fujita, H. (1956) *J. Chem. Phys. 24*, 1084.

Fujita, H. (1962) *"The mathematical theory of sedimentation analysis". Academic Press, New York.*

Garby, L., Gerber, G., and De Verdier (1969) *Europ. J. Biochem. 10*, 110.

Gerhart, J.C., and Schachman, H.K. (1968) *Biochemistry 7*, 538.

Gibbs, J. Willard - *The Collected Works of 1928, Longmans, Green and Co., New York.*

Gilbert, G.A. (1955) *Discuss. Faraday Soc. 20*, 68.

Gilbert, G.A. (1959) *Proc. Roy. Soc., Ser. A 250*, 377.

Gilbert, G.A. (1953) *Proc. Roy. Soc., Ser. A 276*, 354.

Gilbert, G.A. (1966a) *Nature 210*, 299.

Gilbert, G.A. (1966b) *Nature 212*, 296.

Gilbert, G.A., Gilbert, L.M., Owens, C.E., and Shawky, N.A.F. (1972) *Nature New Biology 235*, 110.

Gilbert, L.M, and Gilbert, G.A. (1962) *Nature 194*, 1173.

Glascoe, P.K., and Long, F.A. (1960) *J. Phys. Chem. 64*, 188.

Glazer, A.N., and Smith, E.L. (1960) *J. Biol. Chem. 235*, PC43.

Goldberg, R.J. (1953) *J. Phys. Chem. 57*, 194.

Goldman, R., Silman, H.I., Caplan, S.R., Kedem, O., and Katchalski, E. (1965) *Science 150*, 758.

Greenwald, I. (1925) *J. Biol. Chem. 63*, 339.

Guidotti, G. (1967) *J. Biol. Chem. 242*, 3685.

Hemphill, R.M., Zielke, C.L., and Suelter, C.H. (1971) *J. Biol. Chem. 246*, 7237.

Henderson, R.F., Henderson, T.R., and Woodfin, B.M., (1970) *J. Biol. Chem. 245*, 3733.

Hersh, R., and Schachman, H.K. (1955) *J. Amer. Chem. Soc. 77*, 5228.

Hill, R.J., Konigsberg, W., Guidotti, G., and Craig, L.C. (1962) *J. Biol. Chem. 237*, 1549.

Ho, C., Davis, D.G., Mock, N.H., Lindstrom, T.R., and Charache, S. (1970) *Biochem. Biophys. Res. Commun. 38*, 779.

Howlett, G.J., Jeffrey, P.D., and Nichol, L.W. (1970) *J. Phys. Chem. 74*, 3607.

Howlett, G.J. (1972) *Ph.D. Thesis, University of Melbourne.*

Howlett, G.J., Jeffrey, P.D., and Nichol, L.W. (1972) *J. Phys. Chem. 76*, 777.

Howlett, G.J., and Nichol, L.W. (1972a) *J. Biol. Chem. 247*, 5681.

Howlett, G.J., and Nichol, L.W. (1972b) *J. Phys. Chem. 76*, 2740.

Hvidt, A., and Nielsen, S.O. (1966) *Adv. Protein Chem. 21*, 287.

Inoue, S., Sato, H., Kane, R., and Stephens, R.E. (1965) *J. Cell. Biol. 27*, 115A.

Jeffrey, P.D., and Coates, J.H. (1966) *Biochemistry 5*, 489.

Johnston, J.P., and Ogston, A.G. (1946) *Trans. Faraday Soc. 42*, 789.

Josephs, R., and Harrington, W.F. (1968) *Biochemistry 7*, 2834.

Kauzmann, W. (1959) *Adv. Protein Chem. XIV*, 1.

Kellett, G.L., and Gutfreund, H. (1970) *Nature 227*, 921.

Kirschenbaum, I. (1951) *"Physical properties and analysis of heavy water". McGraw-Hill Book Company, New York.*

Kirschner, K., Eigen, M., Bittman, R., and Voigt, B. (1966) *Proc. Nat. Acad. Sci. U.S.A. 56*, 1161.

Kirschner, K. (1968) *Curr. Topics Microbiol. Immunol. 44*, 123.

Klainer, S.M., and Kegeles, G. (1955) *J. Phys. Chem. 59*, 952.

Klapper, M.H., and Klotz, I.M. (1968) *Biochemistry 7*, 223.

Klotz, I.M. (1946) *Arch. Biochem. 9*, 109.

Klotz, I.M., Walker, F.M., and Pivan, R.B. (1946) *J. Amer. Chem. Soc. 68*, 1486.

Klotz, I.M. (1953) *The Proteins, 1st ed., Vol. IV, Neurath, H., Ed., Academic Press, New York, p. 727.*

Koshland, D.E., Némethy, G., and Filmer, D. (1966) *Biochem. 5*, 365.

Koshland, D.E., and Neet, K.E. (1968) *Ann. Rev. Biochem. 37*, 359.

Kresheck, G.C., Schneider, H., and Scheraga, H.A. (1965) *J. Phys. Chem. 69*, 3132.

Kumosinski, T.F., and Timasheff, S.N. (1966) *J. Amer. Chem. Soc. 88*, 5635.

Kupke, D.W. (1960) *Adv. Protein Chem. 15*, 57.

Langerman, N.R., and Klotz, I.M. (1969) *Biochemistry 8*, 4746.

Lee, J.J., and Berns, D.S. (1968) *Biochem. J. 110*, 465.

Lemberg, R., and Legge, J.W. (1949) *"Hematin Compounds and Bile Pigments: Their Constitution, Metabolism and Function" Interscience, New York, p.228.*

Levitzki, A., and Koshland, D.E. (1969) *Proc. Nat. Acad. Sci. U.S. 62*, 1121.

London, R.E., and Schmidt, P.G. (1972) *Biochemistry 11*, 3136.

Longsworth, L.G., and MacInnes, D.A. (1942) *J. Gen. Physiol. 25*, 507.

Longsworth, L.G. (1943) *J. Amer. Chem. Soc. 65*, 1755.

Longsworth, L.G. (1959) *"Electrophoresis, theory, methods and applications", M. Bier., Ed., Academic Press, New York.*

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193*, 265.

Miller, L.W. (1909) *Z. Phys. Chem. (Leipzig) 69*, 436.

Moffitt, W., and Yang, J.T. (1956) *Proc. Nat. Acad. Sci., U.S. 42*, 596.

Monod, J., Wyman, J., and Changeux, J.P. (1965) *J. Mol. Biol. 12*, 88.

Myer, T.P., and Schellman, J.A. (1962) *Biochim. Biophys. Acta 55*, 361.

McClintock, D.K., and Markus, G. (1969) *J. Biol. Chem. 244*, 36.

McKenzie, H.A., Sawyer, W.H., and Smith, M.B. (1967) *Biochim. Biophys. Acta 147*, 73.

McKenzie, H.A. (1971) in *"Milk Proteins, Chemistry and Molecular Biology". H. A. McKenzie Ed., Vol. 2 Appendix. Academic Press, New York.*

Némethy, G., and Scheraga, H.A. (1964) *J. Chem. Phys., 41*, 680.

Nichol, L.W., and Bethune, J.L. (1963) *Nature 198*, 880.

Nichol, L.W., Bethune, J.L., Kegeles, G., and Hess, E.L. (1964) *"The proteins", Neurath, H., Ed., Vol. 2, Academic Press, New York, p.305.*

Nichol, L.W., and Ogston, A.G. (1965a) *Proc. Roy. Soc. Ser. B 163*, 343.

Nichol, L.W., and Ogston, A.G. (1965b) *J. Phys. Chem. 69*, 1754.

Nichol, L.W., and Ogston, A.G. (1965c) *J. Phys. Chem. 69*, 4365.

Nichol, L.W., Jackson, W.J.H., and Winzor, D.J. (1967a) *Biochemistry 6*, 2449.

Nichol, L.W., Ogston, A.G., and Winzor, D.J. (1967b) *J. Phys. Chem. 71*, 726.

Nichol, L.W., Sawyer, W.H., and Winzor, D.J. (1969) *Biochem. J. 112*, 259.

Nichol, L.W., Smith, G.D., and Winzor, D.J. (1969) *Nature 222*, 174.

Nichol, L.W., Ogston,A.G., and Rescigno, A. (1970) *Proc. Roy. Soc., Ser. A 317*, 153.

Nichol, L.W., Jackson, W.J.H., and Smith, G.D. (1971) *Arch. Biochem. Biophys. 144*, 438.

Nichol, L.W., Jackson, W.J.H., and Winzor, D.J. (1972a) *Biochemistry 11*, 585.

Nichol, L.W., O'Dea, K., and Baghurst, P.A. (1972b) *J. Theoret. Biol. 34*, 255.

Nichol, L.W., and Winzor, D.J. (1972) *"Migration of Interacting Systems" Oxford University Press.*

Nichol, L.W., Janado, M., and Winzor, D.J. (1973) *Biochem. J. (in press).*

Norén, I.B.E., Ho, C., and Casassa, E. (1971) *Biochemistry 10*, 3222.

Ogawa, S., McConnell, H.M., and Horwitz, A. (1968) *Proc. Nat. Acad. Sci. U.S. 61*, 401.

Ogawa, S., and Shulman, R.G. (1971) *Biochem. Biophys. Rev. Commun. 42*, 9.

Ogston, A.G. (1970) *Biochem. J. 119*, 67.

Paglini, S., and Lauffer, M.A., (1968) *Biochemistry 7*, 1827.

Perutz, M.F. (1969) *Proc. Roy. Soc. Ser. B 173*, 113.

Perutz, M.F. (1970a) *Nature 228*, 726.

Perutz, M.F. (1970b) *Nature 228*, 734.

Perutz, M.F. (1972) *Nature 237*, 495.

Porath, J. (1962) *Proc. Int. Symp. Pharm. Chem. 233.*

Poulik, M.D. (1957) *Nature, 180*, 1477.

Rapoport, S., and Guest, G.M. (1941) *J. Biol. Chem. 138*, 269.

Reisler, E., and Eisenberg, H. (1971) *Biochemistry 10*, 2659.

Reithel, F.J. (1963) *Adv. Protein Chem. 18*, 123.

Richards, E.G., and Schachman, H.K. (1959) *J. Phys. Chem. 63*, 1578.

Richards, E.G., Teller, D.C., and Schachman, H.K. (1968) *Biochemistry 7*, 1054.

Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1964) *Adv. Prot. Chem. 19*, 74.

Rubin, M.M., and Changeux, J.P. (1966) *J. Mol. Biol. 21*, 265.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660.

Schmidt, B. (1972) *Angew. Chemie 11*, 576.

Schachman, H.K. (1959) *"Ultracentrifugation in Biochemistry" Academic Press, New York.*

Schachman, H.K., and Edelstein, S.J. (1966) *Biochemistry 5*, 2681.

Smithies, O. (1955) *Biochem. J. 61*, 629.

Stadtman, E.R. (1966) *Advan. Enz. 28*, 41.

Steiner, R.F. (1952) *Arch. Biochem. Biophys. 39*, 33.

Svedberg, T., and Fåhraeus (1926) *J. Amer. Chem. Soc. 48*, 430.

Svedberg, T., and Heyroth, F.F. (1929) *J. Amer. Chem. Soc. 51*, 539.

Svedberg, T., and Hedenius, A. (1934) *Biol. Bull. 66*, 191.

Svedberg, T., and Pedersen, K.O. (1940) *"The ultracentrifuge", Oxford Clarendon Press.*

Tanford, C. (1961) *"Physical Chemistry of Macromolecules", J. Wiley and Sons Inc., New York.*

Timasheff, S.N., and Townend, R. (1969) in *"Protides of the Biological Fluids", Peeters, H., Ed., Pergamon Press, p.33.*

Tombs, M.P. (1957) *Biochem. J. 67*, 517.

Townend, R., and Timasheff, S.N. (1960) *J. Amer. Chem. Soc. 82*, 3168.

Townend, R., Weinberger, L., Timasheff, S.N. (1960) *J. Amer. Chem. Soc. 82*, 3175.

Townend, R., Winterbottom, R.J., and Timasheff, S.N. (1960) *J. Amer. Chem. Soc. 82*, 3161.

Urnes, P., and Doty, P. (1961) *Adv. Protein Chem. 16*, 401.

Weber, G. (1965) in *"Molecular Biophysics", Pullman, B. and Weissbluth, M., Eds., Academic Press, New York, p.369.*

Winzor, D.J., and Scheraga, H.A. (1963) *Biochemistry 2*, 1263.

Winzor, D.J., and Nichol, L.W., (1965) *Biochim. Biophys. Acta 104*, 1.

Winzor, D.J., Loke, J.P. and Nichol, L.W. (1967) *J. Phys. Chem. 71*, 4492.

Witz. J., Timasheff, S.N., and Luzzati, V. (1964) *J. Amer. Chem. Soc. 86*, 168.

Zimmerman, J.K., and Ackers, G.K. (1971) *J. Biol. Chem. 246*, 1078.

Zimmerman, J.K., Cox, D.J., and Ackers, G.K. (1971) *J. Biol. Chem. 246*, 4242.

LIST OF PUBLICATIONS

Sections of the following publications were used in this work:

1. "The Use of Differential Gel Chromatography to Study the Effect of Environment on Protein Polymerizations", Baghurst, P.A., Nichol, L.W., Sawyer, W.H., and Winzor, D.J. (1971) Proc. Aust. Biochem. Soc. 4, 25.

2. "Differential Chromatographic Study of Macromolecular Changes Governed by Environmental Factors", Baghurst, P.A., Nichol, L.W., Richards, R.J. and Winzor, D.J. (1971) Nature 234, 299.

3. "Binding of Dissimilar Ligand Molecules to an Interacting Acceptor: A Model for the Action of Effectors", Nichol, L.W., O'Dea, K., and Baghurst, P.A. (1972) J. Theor. Biol. 34, 255.

4. "The Effect of D_2O on the Association of β-lactoglobulin A", Baghurst, P.A., Nichol, L.W., and Sawyer, W.H. (1972) J. Biol. Chem. 247, 3199.

(*Reprinted from Nature, Vol.* 234, *No.* 5327, *pp.* 299–301, *December* 3, 1971)

Differential Chromatographic Study of Macromolecular Changes governed by Environmental Factors

GILBERT[1] developed a differential gel chromatographic technique to compare the extents of association of two different haemoglobin species. This entailed the layering of a relatively large volume of solution of one species onto a column equilibrated with an identical concentration of the other. Differences in the weight-average elution volumes of the two solutes resulted in a change of protein concentration at the junction of the two solutions, this effect being a consequence of mass conservation. We wish to explore Gilbert's suggestion[1] that the differential method could be adapted to study the effects of environmental factors (*p*H and ionic strength, I) on the state of a single solute system. The acid expansion of bovine serum albumin provides an example where a variation in *p*H induces a change in conformation and thus in elution volume. The effect of ionic strength on the extent of association of β-lactoglobulin A has also been studied, the change of weight-average elution volume in this instance being used to compare the association constants pertinent to two environments of different ionic strength.

To a 1.8 × 19.6 cm column of 'Sephadex G-100', thermostated at 20° C and equilibrated with 0.1 I buffer, *p*H 2 (0.01 M HCl, 0.09 M NaCl) was added 60 ml. of bovine serum albumin (0.94 mg/ml.) dissolved in 0.1 I buffer, *p*H 4.6 (0.01 M sodium acetate, 0.01 M acetic acid, 0.09 M NaCl). The eluate from the column was monitored spectrophotometrically at 280 nm and the volume of fractions was determined accurately by weight. The same method was used to obtain the elution profile in an experiment wherein 35.1 ml. of β-lactoglobulin A (9.81 mg/ml.) in 0.1 I buffer, *p*H 4.65 (0.1 M sodium acetate, 0.09 M acetic acid) was applied to a 1.3 × 23.8 cm column of 'Sephadex G-75', thermostated at 4° C and equilibrated with 0.5 I buffer of the same *p*H (0.1 M sodium acetate, 0.09 M acetic acid, 0.4 M NaCl).

The results obtained in the experiment with bovine serum albumin are shown in Fig. 1*a* from which it is evident that the profile of protein concentration (———) has two distinct plateau regions, the initial elution of protein being at a concentration less than that applied ($\bar{c}^{\alpha}$). The concentration gradient separating the two plateaux coincides with the elution of the proton gradient measured separately and shown by the broken line in Fig. 1*a* (relevant ordinate scale on right). It is noted that the experimental procedure differs from that used

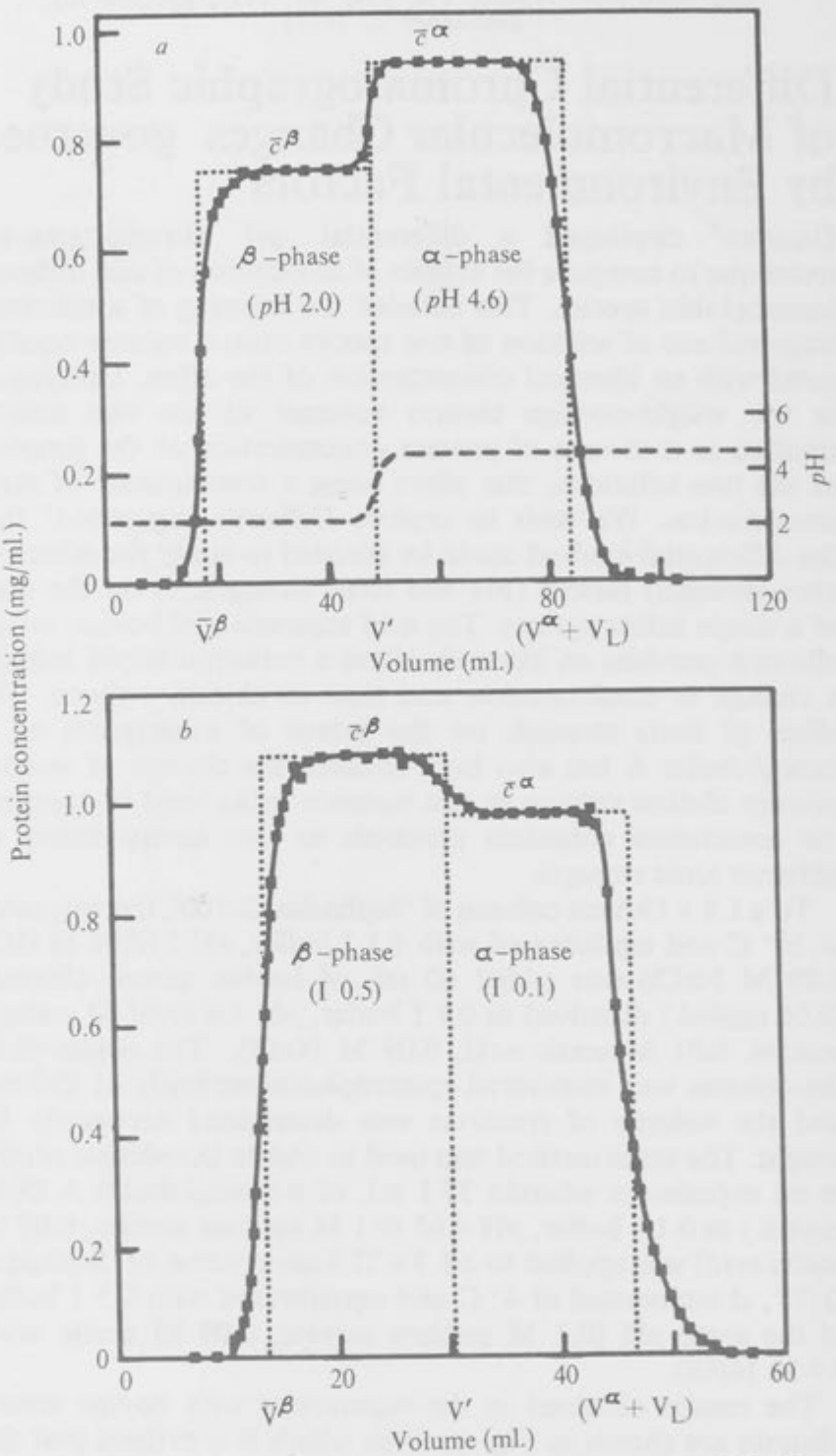


Fig. 1 Elution profiles resulting from (*a*) application of 60 ml. of bovine serum albumin (0.94 mg/ml.) in acetate-chloride buffer (*p*H 4.6) to a 50-ml. column of 'Sephadex G-100' equilibrated with *p*H 2 buffer, and (*b*) application of 35.1 ml. of β-lactoglobulin A (9.81 mg/ml.) in 0.1 I acetate (*p*H 4.65) to a 30-ml. column of 'Sephadex G-75' equilibrated with 0.5 I acetate-chloride buffer (*p*H 4.65). Temperature: *a*, 20° C; *b*, 4° C. Flow rate of each column: 30 ml./h.

by Gilbert[1] inasmuch that prior equilibration of the column with protein is not required: in the present design, the plateau of protein in the *p*H 2 medium is created during the experiment, because of its faster migration rate compared with that of the hydrogen ions. The difference between the two observed plateau concentrations reflects the faster migration rate (smaller elution volume) of bovine serum albumin in the more acidic environment where unfolding has occurred[2]. In the experiment conducted with β-lactoglobulin A (Fig. 1*b*) two plateaux are also observed, but in this case the initial plateau exceeds the applied concentration. This implies that the protein acquires a smaller velocity (larger weight-average elution volume) on passing through the junction between the environments of different ionic strength. Both results are consistent with the following geometric interpretation. The dotted lines in Fig. 1 are median bisectors of observed concentration gradients and permit representation of irregular outlines as rectangles of equivalent area. The total amount of solute added to the column is $\bar{c}^{\alpha}V_L$, where V_L is the loading volume, and for mass to be conserved equals the sum of the areas of the rectangles shown in Fig. 1. Thus

$$\bar{c}^{\alpha}V_L = (V' - \bar{V}^{\beta})\bar{c}^{\beta} + (\bar{V}^{\alpha} + V_L - V')\bar{c}^{\alpha} \qquad (1)$$

wnich on rearrangement yields

$$\frac{\bar{c}^{\alpha}}{\bar{c}^{\beta}} = \frac{V' - \bar{V}^{\beta}}{V' - \bar{V}^{\alpha}} \qquad (2)$$

where bar superscripts denote constituent quantities[3]. Equation (2) is the Johnston-Ogston equation[4], with V' identified as the elution volume of the median bisector of the protein constituent gradient[5].

In relation to Fig. 1*a*, the following values of the parameters in equation (2) pertain: $\bar{c}^{\alpha} = 0.94$ mg/ml., $V' = 48.8$ ml., $\bar{V}^{\beta} = 17.7$ ml., $\bar{V}^{\alpha} = 24.0$ ml. Their substitution in equation (2) yields a value of 0.75 mg/ml. for $\bar{c}^{\beta}$, in complete agreement with that observed experimentally. In a reverse situation where the column was equilibrated with *p*H 4.6 buffer and bovine serum albumin loaded in a *p*H 2 environment, the condition $\bar{V}^{\alpha} < \bar{V}^{\beta}$ pertained and it was observed that $\bar{c}^{\beta} > \bar{c}^{\alpha}$, the result again being in quantitative accord with equation (2). In connexion with the result obtained with β-lactoglobulin A, summarized in Fig. 1*b*, excellent agreement was again found between observed elution volumes (estimated by first moment calculations[6]) and those predicted on the basis of equation (2), the values being $\bar{V}^{\beta} = 13.5$ ml. and $\bar{V}^{\alpha} = 11.5$ ml. In *p*H 4.65, 0.1 I buffer at 4° C, β-lactoglobulin A seems to exist in solution as an equilibrium mixture of essentially monomer, A (molecular weight 36,000), and tetramer, C (molecular weight 144,000), characterized by an association constant, K, of 1.08×10^{-2} l.^{3} g^{-3} (compare ref. 7, which cites earlier references). The definitions

of the equilibrium constant and weight-average elution volume yield the following simultaneous equations.

$$K\,(c_A^{\alpha})^4 + c_A^{\alpha} - \bar{c}^{\alpha} = 0 \qquad (3)$$

$$\overline{V}^{\alpha}\,\bar{c}^{\alpha} = V_A\,c_A^{\alpha} + V_C\,(\bar{c}^{\alpha} - c_A^{\alpha}) \qquad (4)$$

where c_A^{α} is the equilibrium concentration of monomer in the α-phase and V_A and V_C are the elution volumes of the monomer and tetramer species, respectively. With V_C taken as the void volume of the column (9.3 ml.), equations (3) and (4) were solved for the remaining unknown, $V_A = 13.9$ ml. Equations (3) and (4) may be written for the β-phase, whereupon c_A^{β} and K for the 0.5 I environment may be calculated, provided it is assumed that the individual values of V_A and V_C pertain to both α and β phases. A value of 1.0×10^{-4} l.^{3} g^{-3} was found for K in the 0.5 I medium, which clearly shows that an increase of ionic strength promotes the dissociation of tetrameric β-lactoglobulin A.

In summary, the two systems studied illustrate the potential of the differential method for detecting changes in the shape or extent of association of a single solute consequent upon a suitable change of environment. The major advantage of the method is that the change is detected not only by the difference between the observed elution volumes in the two environments ($\overline{V}^{\alpha}$ and $\overline{V}^{\beta}$), but also by the existence of two plateau regions separated by a concentration gradient, the direction of which specifies immediately the relative magnitudes of $\overline{V}^{\alpha}$ and $\overline{V}^{\beta}$. The method should be of particular value for the study, in a wide range of environments, of polymerizing systems which have been previously characterized by a variety of methods under one set of environmental conditions.

P. A. Baghurst
L. W. Nichol

Department of Physical Biochemistry,
John Curtin School of Medical Research,
Australian National University, Canberra

R. J. Richards
D. J. Winzor

Department of Biochemistry,
University of Queensland

Received June 14, 1971.

[1] Gilbert, G. A., *Nature*, **212**, 296 (1966).
[2] Bro, P., Singer, S. J., and Sturtevant, J. M., *J. Amer. Chem. Soc.*, **77**, 4924 (1955).
[3] Tiselius, A., *Nova Acta Soc. Sci. Upsal.*, iv, No. 4, 1 (1930).
[4] Johnston, J. P., and Ogston, A. G., *Trans. Faraday Soc.*, **42**, 789 (1946).
[5] Nichol, L. W., and Ogston, A. G., *J. Phys. Chem.*, **69**, 1754 (1965).
[6] Longsworth, L. G., *J. Amer. Chem. Soc.*, **65**, 1755 (1943).
[7] McKenzie, H. A., *Adv. Protein Chem.*, **22**, 165 (1967).

Printed in Great Britain by Flarepath Printers Limited, Watling Street, Colney Street, St. Albans, Herts.

J. theor. Biol. (1972) **34,** 255–263

Binding of Dissimilar Ligand Molecules to an Interacting Acceptor: A Model for the Action of Effectors

L. W. NICHOL, K. O'DEA AND P. A. BAGHURST

Department of Physical Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T., Australia

and

Department of Biochemistry, University of Melbourne, Parkville, Victoria, Australia

(*Received* 16 *March* 1971, *and in revised form* 25 *May* 1971)

An equation is derived describing the binding of different ligands to distinct sets of equivalent and independent sites on an acceptor, which exists in solution as an equilibrium mixture of isomeric or polymeric forms. It is shown that under certain specified conditions, the binding of one ligand (termed an effector) may perturb the binding of the other. The required simultaneous equations are presented to permit simulation of situations where the effector operates either as an activator or as an inhibitor. It is suggested that these equations may form the basis for curve-fitting experimental binding results obtained with such systems.

1. Introduction

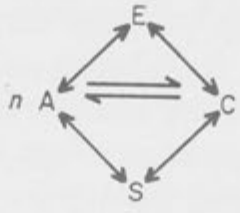
Nichol, Jackson & Winzor (1967) considered the binding of a single type of ligand, S, to an acceptor system which was capable of self-interacting according to the reaction, $nA \rightleftharpoons C$ ($n = 1$, isomerization; $n > 1$, polymerization). A binding equation, describing the relation between a binding function, r (grams of ligand bound per gram of acceptor) and the equilibrium concentration of unbound ligand, [S], was derived in terms of the number of binding sites per molecule of A and C (p and q, respectively) and intrinsic binding constants (Klotz, 1946). Provided the p sites on A are equivalent and independent with respect to the binding of S, a single intrinsic binding constant K_A suffices to describe the multiple binding of S to A: similarly, the intrinsic binding constant K_C characterizes the equilibria involved in the binding of S to q equivalent and independent sites on C. Inspection of the binding equation showed that when $q = np$ and $K_A = K_C$, a plot of r vs. [S] would yield a rectangular hyperbola; whereas when $q \neq np$ and/or $K_A \neq K_C$

(preferential binding of the ligand to one form of the acceptor), the plot would be of sigmoidal form. The result was entirely consistent with that obtained earlier by Monod, Wyman & Changeux (1965) who considered the special case of acceptor isomerization ($n = 1$) in relation to systems associated with sigmoidal binding (or kinetic) curves.

Nichol, Smith & Ogston (1969) noted that several biologically important ligands were themselves capable of self-interaction, and derived binding equations for systems in which both ligand and acceptor, or ligand alone, self-interacted, according to $m\mathrm{S} \rightleftharpoons \mathrm{T}$. They considered only complexes of the type $\mathrm{AS}_i\mathrm{T}_j$ ($i = 0, 1, 2, \ldots p$; $j = 0, 1, 2, \ldots p$; $0 \leqslant i+j \leqslant p$) and $\mathrm{CS}_k\mathrm{T}_l$ ($k = 0, 1, 2, \ldots q$; $l = 0, 1, 2, \ldots q$; $0 \leqslant k+l \leqslant q$) and thereby specified that the different ligand species S and T competed for the *same p* sites on A and the *same q* sites on C. Therefore, their treatment did not encompass situations where two unrelated ligand species S and E, bind to different sites on A and also to distinct sites on C. The problem is of considerable interest since it has been established with certain enzyme systems (see, for example, Gerhart & Pardee, 1962; Changeux, Gerhart & Schachman, 1968), that addition of a ligand (termed an effector) of entirely different structure from that of a substrate and binding away from the active site may modify the form of the sigmoidal curve found in the absence of the effector. It is the purpose of this work to present a generalized approach to the formulation of binding equations describing systems involving a self-interacting acceptor and two types of ligand binding at different sites. Earlier treatments of this problem (Rubin & Changeux, 1966; Frieden, 1967; Changeux & Rubin, 1968) gave no general derivation of binding equations and were aimed at elucidating the action of effectors for the special case of acceptor isomerization ($n = 1$). Whitehead (1970) has provided an elegant treatment of systems in which binding of effectors is related to subunit interactions in a non-polymerizing oligomer.

2. Formulation of the Binding Equation

Consider the model



where ($\leftrightarrow$) denotes multiple binding of ligands S and E to the acceptor species

A and C in equilibrium. For example, with respect to the acceptor species A the following binding equilibria pertain,

$$\begin{array}{lll} A+S \rightleftharpoons AS & ; & A+E \rightleftharpoons AE \quad ; \\ AS_{i-1}+S \rightleftharpoons AS_i & ; & AE_{j-1}+E \rightleftharpoons AE_j \quad ; \\ AS_{p-1}+S \rightleftharpoons AS_p & ; & AE_{w-1}+E \rightleftharpoons AE_w ; \end{array}$$

$$AS_{i-1}E_j+S \rightleftharpoons AS_iE_j;$$

$$AS_iE_{j-1}+E \rightleftharpoons AS_iE_j,$$

where there are p sites per molecule of A which bind S ($i = 1, 2 \ldots p$) and w sites which bind E ($j = 1, 2 \ldots w$). Each equilibrium involving complex formation is (apart from statistical effects) described by a single intrinsic binding constant (Klotz, 1946), which is defined by

$$K_A = \frac{[AS_iE_j]}{[AS_{i-1}E_j][S]}, \tag{1a}$$

$$N_A = \frac{[AS_iE_j]}{[AS_iE_{j-1}][E]}. \tag{1b}$$

Equation (1a) implies that the p sites are equivalent and independent with respect to the binding of S, while equation (1b) similarly assumes equivalence and independence of the w sites binding E. An analogous set of equilibria may be written to describe the multiple binding of S to q sites on the acceptor species C, and of E to y sites on C, whereupon the additional intrisinc binding constants may be defined by

$$K_C = \frac{[CS_kE_l]}{[CS_{k-1}E_l][S]}, \tag{1c}$$

$$N_C = \frac{[CS_kE_l]}{[CS_kE_{l-1}][E]}, \tag{1d}$$

where $k = 1, 2 \ldots q$ and $l = 1, 2 \ldots y$.

In any mixture at equilibrium there will coexist unbound S and E and the complexes AS_iE_j and CS_kE_l, where $i = 0$, $j = 0$ is used to denote uncomplexed A and $k = 0$, $l = 0$ refers to uncomplexed C. The total concentration (g/l) of acceptor, $\bar{c}_A$, initially introduced into the mixture may be written in terms of the molecular weight of A, M_A, the molar concentrations of the complexes, $[AS_iE_j]$ and $[CS_kE_l]$ and the statistical weight of each complex in the mixture (Nichol *et al.*, 1969). The molar concentration, $[AS_iE_j]$, refers to that of a single species rather than to the sum of concentrations of all species of this composition, a similar interpretation being given $[CS_kE_l]$. Therefore

we may write,

$$\bar{c}_A = M_A\left\{\sum_{j=0}^{w}\sum_{i=0}^{p}[AS_iE_j]\alpha_i\beta_j + n\sum_{l=0}^{y}\sum_{k=0}^{q}[CS_kE_l]\alpha_k\beta_l\right\}, \quad (2)$$

where the statistical weights (combinations) are given by

$$\alpha_i = p!/(i!(p-i)!),$$
$$\beta_j = w!/(j!(w-j)!),$$
$$\alpha_k = q!/(k!(q-k)!),$$
$$\beta_l = y!/(l!(y-l)!).$$

Equation (2) may be reformulated employing the intrinsic binding constants (units, mol^{-1}) defined in equation (1):

$$\bar{c}_A = M_A\left\{\sum_{j=0}^{w}\sum_{i=0}^{p}[A](K_A[S])^i\alpha_i(N_A[E])^j\beta_j + \right.$$
$$\left. + n\sum_{l=0}^{y}\sum_{k=0}^{q}[C](K_C[S])^k\alpha_k(N_C[E])^l\beta_l\right\}, \quad (3)$$

where [ ] denote molar concentrations as before. Since α_i, β_j, α_k, β_l are the coefficients of a binomial expansion, equation (3) may be rewritten as,

$$\bar{c}_A = M_A\{[A](1+K_A[S])^p(1+N_A[E])^w + $$
$$+ n[C](1+K_C[S])^q(1+N_C[E])^y\}. \quad (4)$$

Similarly, an expression may be written for the total concentration of S of molecular weight M_S, introduced into the mixture, $\bar{c}_S$ (g/l).

$$\bar{c}_S = M_S[S] + M_S\left\{\sum_{j=0}^{w}\sum_{i=1}^{p} i[AS_iE_j]\alpha_i\beta_j + \sum_{l=0}^{y}\sum_{k=1}^{q} k[CS_kE_l]\alpha_k\beta_l\right\}, \quad (5)$$

where the limits of the summations are chosen to exclude terms involving unbound A and C and complexes involving acceptor and E alone. It follows that

$$\bar{c}_S - M_S[S] = M_S\left\{pK_A[A][S]\sum_{j=0}^{w}\sum_{i=1}^{p}(N_A[E])^j\beta_j(K_A[S])^{i-1}\frac{(p-1)!}{(i-1)!(p-i)!} + \right.$$
$$\left. + qK_C[C][S]\sum_{l=0}^{y}\sum_{k=1}^{q}(N_C[E])^l\beta_l(K_C[S])^{k-1}\frac{(q-1)!}{(k-1)!(q-k)!}\right\}$$
$$= M_S\{pK_A[A][S](1+N_A[E])^w(1+K_A[S])^{p-1} +$$
$$+ qK_C[C][S](1+N_C[E])^y(1+K_C[S])^{q-1}\}. \quad (6)$$

Since S and E are merely symbols, it follows by analogy with equation (6) that the total weight concentration of E is given by,

$$\bar{c}_E - M_E[E] = M_E\{wN_A[A][E](1+K_A[S])^p(1+N_A[E])^{w-1} +$$
$$+ yN_C[C][E](1+K_C[S])^q(1+N_C[E])^{y-1}\}. \quad (7)$$

A binding function may be defined as *either* $(\bar{c}_S - M_S[S])/\bar{c}_A$ *or* $(\bar{c}_E - M_E[E])/\bar{c}_A$

and we lose no generality by considering only the former. In these terms, combination of equations (4) and (6) gives the binding equation,

$$r = \frac{pM_S K_A[A][S](1+N_A[E])^w(1+K_A[S])^{p-1} + qM_S K_C[C][S](1+N_C[E])^y(1+K_C[S])^{q-1}}{M_A[A](1+K_A[S])^p(1+N_A[E])^w + nM_A[C](1+K_C[S])^q(1+N_C[E])^y}. \quad (8)$$

3. Discussion of the Binding Equation

(A) CASES WHERE *E* DOES NOT FUNCTION AS AN EFFECTOR

When $K_A = K_C$, $q = np$, $N_A = N_C$ and $y = nw$, then equation (8) may be factorized and simplified to yield

$$r = pM_S K_A[S]/M_A(1+K_A[S]). \quad (9)$$

Thus, in this case the binding curve obtained by studying the amount of S bound in the presence of E is the same rectangular hyperbola as that obtained in studying the binding of ligand to equivalent and independent sites on a non-self-interacting acceptor in the absence of E. It is convenient for what follows to view this situation in an alternate way. The total molar concentration of A in all forms in the equilibrium mixture is given by,

$$[\bar{A}] = [A](1+K_A[S])^p(1+N_A[E])^w. \quad (10)$$

Similarly,

$$[\bar{C}] = [C](1+K_C[S])^q(1+N_C[E])^y, \quad (11)$$

where $\bar{c}_A = M_A[\bar{A}]+nM_A[\bar{C}]$. Therefore, we may form the ratio,

$$\frac{[\bar{C}]}{[\bar{A}]^n} = \frac{[C](1+K_C[S])^q(1+N_C[E])^y}{[A]^n(1+K_A[S])^{np}(1+N_A[E])^{nw}}. \quad (12)$$

Clearly, in the situation under discussion ($K_A = K_C$, $q = np$, $N_A = N_C$ $y = nw$) it follows from equation (12) that $[\bar{C}]/[\bar{A}]^n = [C]/[A]^n = X$, the equilibrium constant governing the acceptor interaction, $nA \rightleftharpoons C$. It is also evident from equation (12) that when $N_A = N_C$, $y = nw$, $K_A \neq K_C$ and/or $q \neq np$, the ratio $[\bar{C}]/[\bar{A}]^n$ is no longer a function of [E]: in this case E is not an effector in the binding of S, which itself is described by a sigmoidal binding curve.

(B) CASES WHERE *E* ACTS AS AN EFFECTOR

The remaining possibilities are encompassed by the conditions $q \neq np$ and/or $K_A \neq K_C$ and $y \neq nw$ and/or $N_A \neq N_C$. It is immediately apparent from equation (12) that $[\bar{C}]/[\bar{A}]^n$ is a function of both [S] and [E]. Thus, the binding of one ligand must perturb the binding of the other. For the special case $n = 1$ (acceptor isomerization), the right hand side of equation (8) may

be divided by $[A](1+N_A[E])^w$ to give

$$r = \frac{pM_SK_A[S](1+K_A[S])^{p-1} + qM_SK_C[S](1+K_C[S])^{q-1}X'}{M_A(1+K_A[S])^p + M_A(1+K_C[S])^qX'}$$

where

$$X' = [C](1+N_C[E])^y/[A](1+N_A[E])^w \qquad (13)$$

Rubin & Changeux (1966), Changeux & Rubin (1968) and Frieden (1967) reported the same result for $n = 1$, but in terming X' an apparent conformational equilibrium constant, failed to stress its functional relation to the concentration of unbound effector, [E]. The importance of this point is well illustrated with a numerical example. Corresponding values of r for a range of values of [S] may be computed from equation (13) for a system of specified parameters, including $X = [C]/[A]$ (for $n = 1$), only if corresponding values of [E] may be determined. Any two of equations (4), (6) and (7) may be solved simultaneously to find the required values of [E] for selected [S] values. An illustration is provided in Fig. 1 for arbitrarily selected values of the parameters reported therein. It is clear from Fig. 1 that a family of binding

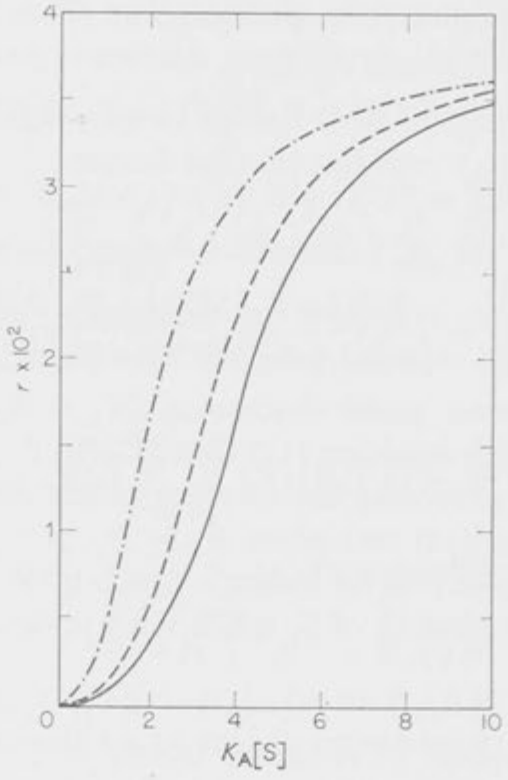


FIG. 1. Computed binding curves for a system in which the acceptor isomerizes, (A $\rightleftarrows$ C), with ligand S binding to the A isomer and effector E binding to the C isomer. The family of curves show an inhibitory effect of E increasing with total concentration of E included in the reaction mixture. Selected values of the parameters (see text) were as follows: $M_S = 200$, $M_A = 20{,}000$, $M_E = 200$, $X = 50$, $\bar{c}_{A_t} = 10$ g/l, $p = 4$, q and/or $K_C = 0$, $y = 1$, $N_C = 1 \times 10^2$ l/mol, w and/or $N_A = 0$, $\bar{c}_E = 0$ g/l (–·–·–); 10 g/l (– – – –); 20 g/l (———).

curves may result from systematically varying $\bar{c}_E$ holding other parameters fixed. In this case, the degree of sigmoidality increases with increasing $\bar{c}_E$ and the effector, E, is termed an inhibitor. It may similarly be shown that, if S binds preferentially to C (not necessarily exclusively) and E binds preferentially to A, that again E acts as an inhibitor. A situation in which ligand S, and effector E, both bind preferentially to the same acceptor species results in sigmoidal curves tending to assume the form of the limiting rectangular hyperbola as $\bar{c}_E$ increases (numerical examples not shown). In the latter case, E would be termed an activator.

Equation (8) also applies when the monomer of acceptor coexists in equilibrium with a higher polymeric form ($n > 1$) and again a set of simultaneous equations may be solved to provide numerical examples. Since $[C] = X[A]^n$ it is clear that the simultaneous equations are polynomials, but (for example) with $n = 2$ (dimerizing acceptor) the solution, accepting only positive real roots, is not difficult. Figure 2 illustrates situations where ligand S binds preferentially to A with E absent (– – – –), with E acting as activator by also binding preferentially to A (–·–·–) and with E acting as an inhibitor by binding preferentially to C (——).

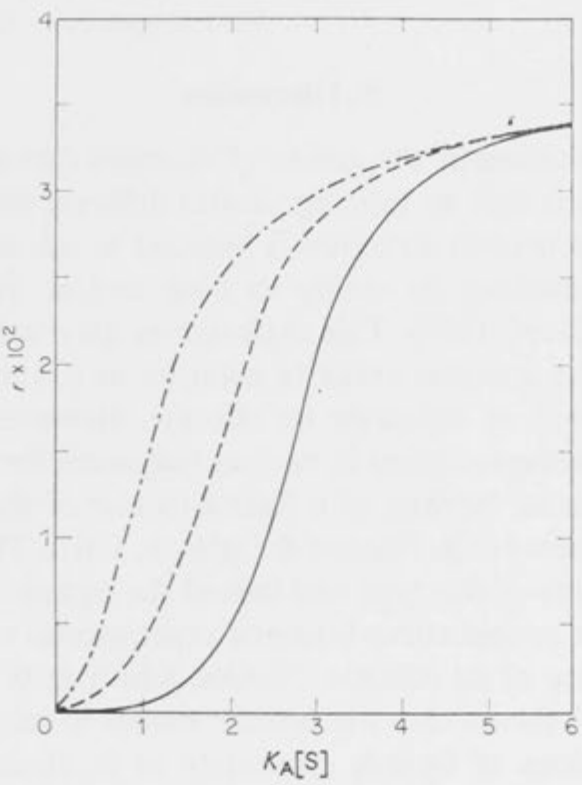


FIG. 2. Computed binding curves for systems involving a dimerizing acceptor ($n = 2$). In each case $M_S = 200$, $M_A = 20{,}000$, $M_E = 200$, $X = 5 \times 10^6$ l/mol, $\bar{c}_A = 10$ g/l, $p = 4$, q and/or $K_C = 0$. – – – –, $\bar{c}_E = 0$, effector is absent; –·–·–, $\bar{c}_E = 10$ g/l, $w = 1$, $N_A = 10^2$ l/mol, y and/or $N_C = 0$, effector is an activator; ———, $\bar{c}_E = 10$ g/l, w and/or $N_A = 0$, $y = 1$, $N_C = 10^2$ l/mol, effector is an inhibitor.

4. Generalization of the Binding Equation

In a more general case where a series of polymeric forms of acceptor coexist in equilibrium, each capable of binding ligand S and a variety of effector species (the set of sites for a particular effector being distinct from all other ligand binding sites), it may be shown that

$$r = \frac{M_S \sum_m [m][S]\tau_{m,S} K_{m,S}(1+K_{m,S}[S])^{\tau_{m,S}-1} \prod_x (1+K_{m,x}[x])^{\tau_{m,x}}}{M_1 \sum_m m[m](1+K_{m,S}[S])^{\tau_{m,S}} \prod_x (1+K_{m,x}[x])^{\tau_{m,x}}}, \quad (14)$$

where m refers to the acceptor species ($m = 1$, monomer of molecular weight, M_1; $m = 2$, dimer, etc.), $[m]$ represents the free molar concentration of that polymer, $K_{m,S}$ the intrinsic binding constant of S to that polymer, and $\tau_{m,S}$ the number of binding sites per molecule of the respective polymer. In addition, in equation (14) $K_{m,x}$ denotes the intrinsic binding constant of the xth effector to the mth acceptor species, $\tau_{m,x}$ the corresponding number of binding sites and $[x]$ the molar concentration of unbound xth effector. Equation (14) encompasses the quite realistic situation where both activators and inhibitors may perturb the form of the binding curve found with S alone.

5. Discussion

One direct explanation of the action of effectors (activators or inhibitors) on enzyme systems is that on binding to sites different from the active site, a perturbation of the protein structure is induced which affects the active site enhancing or diminishing its ability to bind and/or breakdown substrate (e.g. Koshland & Neet, 1968). This explanation may well suffice to describe systems wherein the acceptor exists in solution as a non-interacting species prior to the addition of substrate or effector. However, cases are known where an enzymic acceptor exists in various polymeric forms in the absence of ligand and preferential binding of a ligand to one of the forms results in a sigmoidal binding curve (e.g. Frieden & Colman, 1967). The present treatment is relevant to systems of this type and indeed the equations developed herein are essential for the proper curve-fitting of experimental results obtained with them in the presence of an effector (Nichol, Smith & Winzor, 1969). Moreover, equation (12) shows that the present model is subject to experimental test, in that additions of ligands separately or in chosen combination are expected to alter the distribution of macromolecular acceptor species (bound and unbound forms). In the case of a polymerizing acceptor such alterations may be examined in terms of a weight-average property of the system (Sophianopolous, 1969; Hoagland & Teller, 1969). Finally, it is noted that the

condition for E to act as an effector in the binding of S, viz. $q \neq np$ and/or $K_A \neq K_C$ and $y \neq nw$ and/or $N_A \neq N_C$, is not physically unreal. Thus, binding sites may be masked in polymer formation and/or conformational changes (possibly induced in polymer formation in the absence of any added ligand) may affect the different sets of binding sites and hence intrinsic binding constants in a way used in the present development.

REFERENCES

CHANGEUX, J.-P. & RUBIN, M. M. (1968). *Biochemistry* **7,** 538.
CHANGEUX, J.-P., GERHART, J. C. & SCHACHMAN, H. K. (1968). *Biochemistry* **7,** 531.
FRIEDEN, C. (1967). *J. biol. Chem.* **242,** 4045.
FRIEDEN, C. & COLMAN, R. F. (1967). *J. biol. Chem.* **242,** 1705.
GERHART, J. C. & PARDEE, A. B. (1962). *J. biol. Chem.* **237,** 891.
HOAGLAND, V. D. & TELLER, D. C. (1969). *Biochemistry* **8,** 594.
KLOTZ, I. M. (1946). *Archs Biochem. Biophys.* **9,** 109.
KOSHLAND, D. E. & NEET, K. E. (1968). *A. Rev. Biochem.* **37,** 359.
MONOD, J., WYMAN, J. & CHANGEUX, J.-P. (1965). *J. molec. Biol.* **12,** 88.
NICHOL, L. W., JACKSON, W. J. H. & WINZOR, D. J. (1967). *Biochemistry* **6,** 2449.
NICHOL, L. W., SMITH, G. D. & OGSTON, A. G. (1969). *Biochim. biophys. Acta* **184,** 1.
NICHOL, L. W., SMITH, G. D. & WINZOR, D. J. (1969). *Nature, Lond.* **222,** 174.
RUBIN, M. M. & CHANGEUX, J.-P. (1966). *J. molec. Biol.* **21,** 265.
SOPHIANOPOULOS, A. J. (1969). *J. biol. Chem.* **244,** 3188.
WHITEHEAD, E. (1970). *Biochemistry* **9,** 1440.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 247, No. 10, Issue of May 25, pp. 3198-3204, 1972
Printed in U.S.A.

The Effect of D_2O on the Association of β-Lactoglobulin A*

(Received for publication, December 28, 1971)

PETER A. BAGHURST, LAWRENCE N. NICHOL, AND WILLIAM H. SAWYER

From the Russell Grimwade School of Biochemistry, University of Melbourne, Parkville, Victoria, Australia, and the Department of Physical Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, Australian Capital Territory, Australia

SUMMARY

The association-dissociation equilibrium of the genetic variant β-lactoglobulin A has been studied in an aqueous environment (acetate buffer, pH 4.65, $\Gamma/2 = 0.1$) and one in which D_2O replaced H_2O (pD 4.65). Sedimentation velocity experiments (analyzed with respect to the area distribution of bimodal reaction boundaries and weight-average sedimentation coefficients) indicated that the extent of association was increased in the heavy water medium. The conclusion was supported by results obtained by Sephadex chromatography and by treatment of the Moffitt-Yang parameter, $\bar{a}_0$, derived from optical rotatory dispersion studies, as a weight-average quantity. The latter method was also used to evaluate enthalpy changes associated with polymer formation in the two environments, the values being −64 and −69 kcal per mole in the H_2O and D_2O media, respectively. The results are compared with the stabilizing effect of D_2O previously reported for other proteins and discussed in terms of increased interactions of both hydrogen bonding and hydrophobic types.

Lee and Berns (1) have suggested that the tendency of deuterium oxide to increase the state of aggregation of certain proteins (2, 3) is due to enhanced hydrophobic interactions (4). The glutamate dehydrogenase system provides an example where it has been suggested (5) that the formation of a series of higher polymers is largely entropically driven, the heats of association being positive. Henderson *et al.* (3) compared values of the apparent weight-average molecular weight of the enzyme found in an aqueous environment (pH 8) and in a medium where D_2O replaced H_2O (pD 8). At each enzyme concentration examined, the results showed that the extent of association was greater in the D_2O medium, a finding in apparent agreement with the hypothesis of Lee and Berns (1), but attributed by Henderson *et al.* (3) to increased strength of deuterium bonds and deuterium water bridges. Before accepting the use of D_2O as a test for the relative importance of hydrophobic interactions in stabilizing various protein aggregates (1), it is desirable to examine its action on polymerizing systems associated with negative enthalpy changes.

* This work was supported in part by the Australian Research Grants Committee.

In this work, the effect of D_2O on the association of the genetic variant β-lactoglobulin A is considered. Although results pertaining to the polymerization behavior of the protein in aqueous media of pH 4.6 have been given various detailed interpretations (6, 7), it appears that the predominant species coexisting in equilibrium are the dimer (molecular weight 36,000) and the octamer (molecular weight 144,000) (8, 9). The enthalpy change associated with the dimer-octamer equilibrium operating in aqueous media has been established as negative (8–10), a variety of evidence (11) suggesting that hydrogen bond formation between aspartyl and glutamyl residues may contribute significantly to octamer formation. Although several different types of interaction may contribute to the association of a given protein, it appears that those involved in the octamer formation of β-lactoglobulin A are not predominantly hydrophobic and accordingly the effect of D_2O on this reasonably well defined system is of interest.

EXPERIMENTAL PROCEDURE

Materials

β-Lactoglobulin A was prepared from the milk of single cows which had been typed homozygous for the A variant by a previously described starch gel electrophoresis method (12). The preparative method was that of Armstrong *et al.* (13). The recrystallized protein was dissolved in either aqueous acetate buffer (0.1 M sodium acetate, 0.088 M acetic acid, pH 4.65, $\Gamma/2 = 0.1$) or a buffer in which the glass-distilled water was replaced by heavy water (International Chemical and Nuclear Corp.). Measurement of pH was made at 20° with a Radiometer pH meter equipped with standard glass and calomel electrodes: with D_2O solutions the meter reading was adjusted to a value of 4.25 with acetic acid, since the pD of D_2O solutions (14) equals the meter reading plus 0.4. The procedure accounts for the difference in the ionization constants of H_2O and D_2O and yields a pD of 4.65. Protein concentrations were measured spectrophotometrically at 278 nm with an absorbance coefficient (15) of 0.96 liter per g per cm for both H_2O and D_2O solutions.

Sedimentation

Sedimentation velocity experiments were conducted in a Spinco model E ultracentrifuge at 59,780 rpm. The temperature was measured and controlled with the RTIC unit. Schlieren patterns were measured with a two-dimensional comparator (Nikon Shadowgraph model 6C) and weight-average sedimentation coefficients were calculated from the rate of

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 247, No. 10, Issue of May 25, pp. 3198–3204, 1972
Printed in U.S.A.

The Effect of D_2O on the Association of β-Lactoglobulin A*

(Received for publication, December 28, 1971)

PETER A. BAGHURST, LAWRENCE N. NICHOL, AND WILLIAM H. SAWYER

From the Russell Grimwade School of Biochemistry, University of Melbourne, Parkville, Victoria, Australia, and the Department of Physical Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, Australian Capital Territory, Australia

SUMMARY

The association-dissociation equilibrium of the genetic variant β-lactoglobulin A has been studied in an aqueous environment (acetate buffer, pH 4.65, Γ/2 = 0.1) and one in which D_2O replaced H_2O (pD 4.65). Sedimentation velocity experiments (analyzed with respect to the area distribution of bimodal reaction boundaries and weight-average sedimentation coefficients) indicated that the extent of association was increased in the heavy water medium. The conclusion was supported by results obtained by Sephadex chromatography and by treatment of the Moffitt-Yang parameter, $\bar{a}_0$, derived from optical rotatory dispersion studies, as a weight-average quantity. The latter method was also used to evaluate enthalpy changes associated with polymer formation in the two environments, the values being −64 and −69 kcal per mole in the H_2O and D_2O media, respectively. The results are compared with the stabilizing effect of D_2O previously reported for other proteins and discussed in terms of increased interactions of both hydrogen bonding and hydrophobic types.

Lee and Berns (1) have suggested that the tendency of deuterium oxide to increase the state of aggregation of certain proteins (2, 3) is due to enhanced hydrophobic interactions (4). The glutamate dehydrogenase system provides an example where it has been suggested (5) that the formation of a series of higher polymers is largely entropically driven, the heats of association being positive. Henderson *et al.* (3) compared values of the apparent weight-average molecular weight of the enzyme found in an aqueous environment (pH 8) and in a medium where D_2O replaced H_2O (pD 8). At each enzyme concentration examined, the results showed that the extent of association was greater in the D_2O medium, a finding in apparent agreement with the hypothesis of Lee and Berns (1), but attributed by Henderson *et al.* (3) to increased strength of deuterium bonds and deuterium water bridges. Before accepting the use of D_2O as a test for the relative importance of hydrophobic interactions in stabilizing various protein aggregates (1), it is desirable to examine its action on polymerizing systems associated with negative enthalpy changes.

* This work was supported in part by the Australian Research Grants Committee.

In this work, the effect of D_2O on the association of the genetic variant β-lactoglobulin A is considered. Although results pertaining to the polymerization behavior of the protein in aqueous media of pH 4.6 have been given various detailed interpretations (6, 7), it appears that the predominant species coexisting in equilibrium are the dimer (molecular weight 36,000) and the octamer (molecular weight 144,000) (8, 9). The enthalpy change associated with the dimer-octamer equilibrium operating in aqueous media has been established as negative (8–10), a variety of evidence (11) suggesting that hydrogen bond formation between aspartyl and glutamyl residues may contribute significantly to octamer formation. Although several different types of interaction may contribute to the association of a given protein, it appears that those involved in the octamer formation of β-lactoglobulin A are not predominantly hydrophobic and accordingly the effect of D_2O on this reasonably well defined system is of interest.

EXPERIMENTAL PROCEDURE

Materials

β-Lactoglobulin A was prepared from the milk of single cows which had been typed homozygous for the A variant by a previously described starch gel electrophoresis method (12). The preparative method was that of Armstrong *et al.* (13). The recrystallized protein was dissolved in either aqueous acetate buffer (0.1 M sodium acetate, 0.088 M acetic acid, pH 4.65, Γ/2 = 0.1) or a buffer in which the glass-distilled water was replaced by heavy water (International Chemical and Nuclear Corp.). Measurement of pH was made at 20° with a Radiometer pH meter equipped with standard glass and calomel electrodes: with D_2O solutions the meter reading was adjusted to a value of 4.25 with acetic acid, since the pD of D_2O solutions (14) equals the meter reading plus 0.4. The procedure accounts for the difference in the ionization constants of H_2O and D_2O and yields a pD of 4.65. Protein concentrations were measured spectrophotometrically at 278 nm with an absorbance coefficient (15) of 0.96 liter per g per cm for both H_2O and D_2O solutions.

Sedimentation

Sedimentation velocity experiments were conducted in a Spinco model E ultracentrifuge at 59,780 rpm. The temperature was measured and controlled with the RTIC unit. Schlieren patterns were measured with a two-dimensional comparator (Nikon Shadowgraph model 6C) and weight-average sedimentation coefficients were calculated from the rate of

movement of the square root of the second moment of the entire bimodal boundary (16). Values were corrected to 20° in water by the procedure outlined by Lee and Berns (1), the required densities and viscosities of H_2O and D_2O at different temperatures being taken from tables presented by Kirshenbaum (17). The partial specific volume of the protein was taken as 0.746 ml per g in water (9), while that for the deuterated protein was obtained by division by the correction factor 1.015 (1, 3). It could be noted that the corrected weight-average values, $\bar{s}_{20,w}$, are hypothetical since the enthalpy change involved in polymerization is finite. In the area analysis of bimodal reaction boundaries, comparator readings were plotted on graph paper with an enlarged scale and the areas of graphically resolved peaks were measured with a planimeter.

Chromatography

Three frontal analysis chromatography experiments (18) were conducted, all employing columns of Sephadex G-75 at 12°. In the first, the aqueous acetate buffer described above was used to equilibrate the column (36 × 1 cm), to dissolve the applied protein (19 ml of a 1 g per dl solution) and to elute. The second experiment was of the same design except that the column dimensions were 38 × 1 cm and the D_2O medium was used throughout. In the third experiment, the column (38 × 1 cm) was equilibrated with the aqueous acetate buffer, while the D_2O medium was used to dissolve the applied protein (0.740 g per dl) and to elute. The latter experiment is of a design termed differential chromatography (19). In each experiment fractions of approximately 0.3 ml were collected in previously tared tubes and the actual volumes were determined by weight employing a suitable density correction. The protein concentration in each fraction was determined spectrophotometrically and by the method of Lowry *et al.* (20).

Optical Rotatory Dispersion

ORD[1] results were obtained with a Perkin-Elmer model 141 spectropolarimeter at the following wave lengths: 578, 546, 436, 405, and 365 nm. A jacketed 10-cm quartz polarimeter cell thermostated at the temperatures specified in the text was employed. The results were analyzed according to the phenomenological equation of Moffitt and Yang (21) employing a value of 212 nm for λ_0 to yield the parameter, a_0, from the ordinate intercept of the linear plots obtained. ORD studies were also performed with a Jasco ORD/UV-5 instrument and a quartz cell (thermostated at 20°) of 2-mm path length. Recordings obtained with protein solutions (0.01 g per dl) in both H_2O and D_2O media were compared in the range 220 to 260 nm: at lower wave lengths the acetate buffer absorbed strongly.

Methods Used to Evaluate Apparent Association Constant, K

Method 1—The sedimentation velocity of a rapidly equilibrating mixture of two polymeric forms (with the exception of a monomer-dimer system) will give rise to a bimodal reaction boundary (22), the concentration corresponding to the area of the back portion of the boundary, Δ_2, being related to K and the degree of polymerization, n, by (23)

$$K = \frac{\Delta_2^{1-n}[2(n^2-1)]^{n-1}[n-2]}{[n(2n-1)]^n} \quad (1)$$

For the dimer-octamer equilibrium under discussion $n = 4$ and the value of K ($l^3 \cdot g^{-3}$) may readily be estimated, although it must be regarded as approximate in view of errors involved in area resolution and measurement.

Method 2—From the definitions of the weight-average sedimentation coefficient of a system $nA \rightleftharpoons C$, and the equilibrium constant, $K = c_C/c_A{}^n$, it may be shown that

$$K = \frac{\bar{c}^{(1-n)}[(s_A)_{T,b} - (s_C)_{T,b}]^{n-1}[(s_A)_{T,b} - \bar{s}_{T,b}]}{[\bar{s}_{T,b} - (s_C)_{T,b}]^n} \quad (2)$$

In Equation 2 $\bar{c}$ is the total weight concentration ($c_A + c_C$), $\bar{s}_{T,b}$ is the corresponding weight-average sedimentation coefficient in the buffer at temperature T and $(s_A)_{T,b}$ and $(s_C)_{T,b}$ are the sedimentation coefficients of the individual forms A and C in the same environment. The right-hand side of Equation 2 may be multiplied throughout by the factor (1) used to correct all sedimentation coefficients to 20° in water without affecting the value of K. This is a convenient procedure since estimates of the values of the sedimentation coefficients of the dimer (A) and the octamer (C) of β-lactoglobulin A at 20° in water and at finite protein concentrations are available (8). However, as these values are themselves based on assumptions concerning the frictional coefficients and concentration dependence coefficients of A and C, the values of K derived from Equation 2 are to this extent uncertain.

Method 3—Winzor *et al.* (24) have described in detail a method by which the weight- and z-average elution volumes may be evaluated from an elution profile obtained in a frontal analysis Sephadex chromatography experiment and used to estimate the elution volumes of the individual species A and C coexisting in rapid equilibrium. In this study Sephadex G-75 was selected to exclude the octamer so that V_C equaled the void volume and V_A could be calculated from individual experiments with Equation 12 of Winzor *et al.* (24). These values may be used in conjunction with the measured weight-average elution volume and the corresponding total concentration, $\bar{c}$, pertaining to the plateau region to calculate K. The required expression is entirely analogous to Equation 2 with elution volumes replacing sedimentation coefficients.

Method 4—McKenzie *et al.* (9) have shown that the treatment of a_0 derived from ORD measurements as a weight-average quantity, $\bar{a}_0$, led to estimates of K for the dimer-octamer equilibrium of β-lactoglobulin A in aqueous acetate buffer (pH 4.6) in agreement with those derived from weight-average molecular weight studies. Their equation (9) is entirely equivalent to Equation 2 written in terms of $\bar{a}_0$ and the value of the parameter for A and C ($a_{0,A}$ and $a_{0,C}$, respectively). In this work, values of $a_{0,A}$ and $a_{0,C}$ in both H_2O and D_2O media were found from plots of $\bar{a}_0$ *versus* temperature in the manner previously described (9).

RESULTS

Sedimentation Velocity Studies—Sedimentation velocity patterns obtained at 12° with solutions of β-lactoglobulin in the aqueous and D_2O media are compared in Fig. 1. It is evident that bimodal reaction boundaries (22) were observed in each experiment and that the relative proportion of the total area associated with the trailing portion of the boundary is greater in the aqueous environment (*upper pattern*). Similar results were obtained at other temperatures and the patterns were analyzed according to Equation 1 to estimate the association

[1] The abbreviation used is: ORD, optical rotatory dispersion.

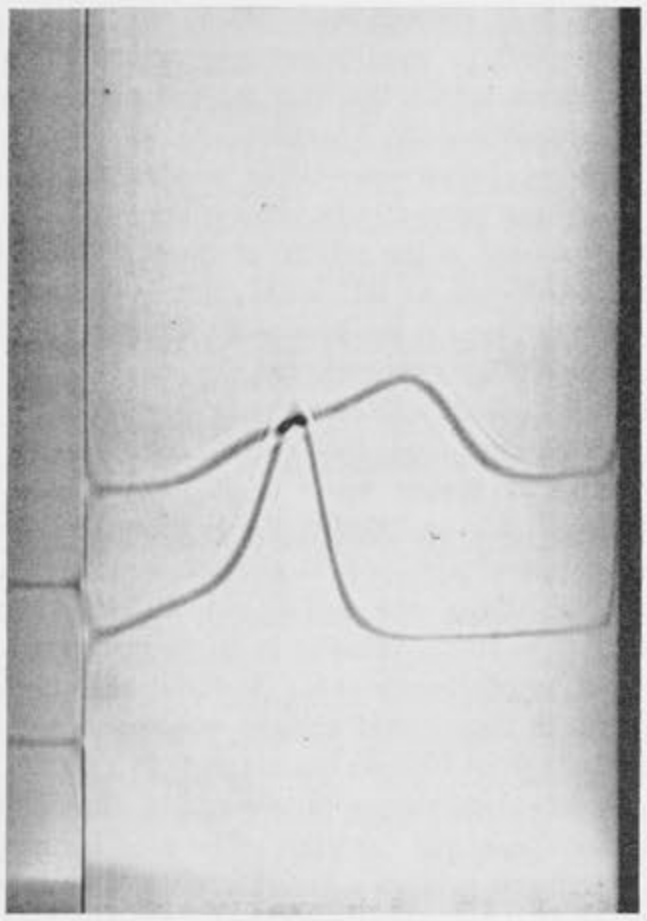


FIG. 1. Sedimentation velocity patterns obtained with solutions of β-lactoglobulin A (1.3 g per dl) at 12°. Sedimentation is from *left* to *right*. The *upper pattern* refers to an aqueous acetate buffer (pH 4.65) and the *lower pattern* to a D_2O medium (pD 4.65).

constant, K and hence log K_a, where K_a is expressed as l^3 base mole^{-3} to permit later comparison with the results of McKenzie *et al.* (9). These values of log K_A found by Method 1 are summarized in Table I. Although the unimodal boundary observed in aqueous acetate buffer at 20° could not be resolved, it is clear from the results in Table I that K_a is greater in the heavy water environment at all temperatures studied and that in both environments it decreases with increasing temperature. These conclusions are supported by the analysis of the same sedimentation velocity results in terms of weight-average sedimentation coefficients (Table I, Method 2). For these calculations Equation 2 was employed together with values of the sedimentation coefficients of dimer and octamer, calculated at finite protein concentration from the data summarized by Gilbert (8). It is noted that the values of log K_a found by Method 2 are consistently lower than those derived from Method 1 (and others to be presented). This may well arise as a consequence of the necessity of estimating the sedimentation coefficient of octamer (8) and of applying large (and essentially arbitrary) correction factors (1) to convert sedimentation coefficients observed under the specified conditions to 20° in water.

Frontal Analysis in Sephadex Chromatography—Fig. 2 presents the elution profile obtained on subjecting the protein to frontal analysis conducted entirely in the D_2O medium. In accordance with theoretical prediction (18, 22–24) for a rapidly equilibrating mixture of polymeric forms, the advancing front is sharper than the trailing boundary and no resolution is observed on either side. The elution volume of the dimer, V_A, was computed from the weight-average elution volume pertaining to the trailing side (V_w = 31.2 ml), the corresponding z-average quantity (V_z = 31.9 ml) and the void volume (11.6 ml) plus the loading volume (17.0 ml), the sum being equivalent to the elution volume of excluded octamer, V_C, on the trailing side. The

TABLE I

Apparent association constants for dimer-octamer equilibrium of β-lactoglobulin A derived from sedimentation velocity experiments

Experiments were conducted in aqueous acetate buffer (pH 4.65) and a deuterium oxide medium (pD 4.65) with a fixed protein concentration of 1.3 g per dl. K_a is expressed in l^3 base mole^{-3}.

Method	Temperature	log K_a	
		H_2O	D_2O
1	4°	11.1	12.2
1	12	10.0	11.3
1	20		10.5
2	4	10.3	10.9
2	12	9.6	10.0
2	20	8.7	9.5

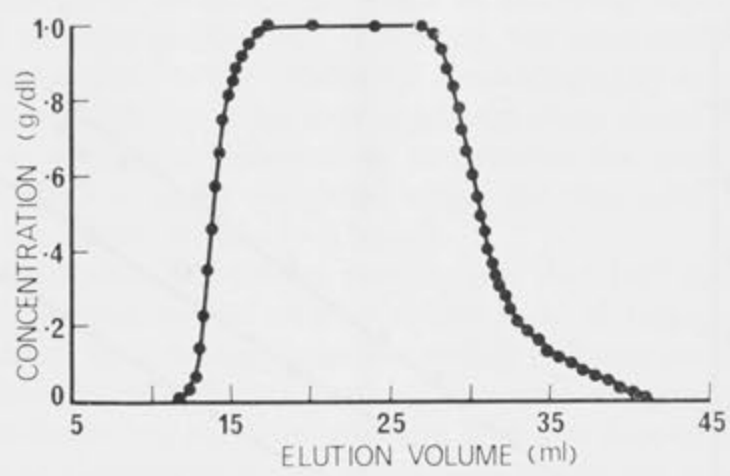


FIG. 2. The elution profile obtained from a frontal analysis chromatography experiment on Sephadex G-75 (38 × 1 cm) conducted at 12°. A D_2O medium (pD 4.65) was used to equilibrate the column and to dissolve the applied β-lactoglobulin A (17 ml of 1 g per dl).

value of V_A obtained (39.6 ml) was then employed together with V_w and V_C to calculate log K_a in D_2O at 12°. The value obtained of 12.5 may be compared with that of 10.9 found by analyzing in a similar manner the frontal analysis experiment conducted entirely in the aqueous acetate buffer at 12°, for which the following elution volumes pertaining to the trailing side were found: V_w = 33.6 ml, V_z = 34.3 ml, V_C = 28.6 ml, and V_A = 38.3 ml. The values of V_A obtained in the two experiments conducted with columns of slightly different size may be compared by transforming the data to corresponding values of K_{av}, the distribution coefficient defined by Andrews (25): in relation to the advancing side, K_{av} (H_2O) = 0.53 and K_{av} (D_2O) = 0.60. Since V_A is a derived quantity, the agreement is reasonable and provides the first indication that the size and shape properties of the dimer of β-lactoglobulin A are not markedly affected by the change of environment. Of greater interest is the observation that Method 3 confirms the conclusion drawn from Methods 1 and 2 that the extent of association of the protein is greater in the D_2O medium.

In the differential chromatography experiment (19) involving the application of protein in the D_2O environment to a column equilibrated with aqueous acetate buffer, it was found in contrast to the results shown in Fig. 2 that two plateaux were evident in the elution profile. The concentration gradient separating the two plateaux occurred at an elution volume, V' = 33.0 ml, which also corresponded to the median bisector of the

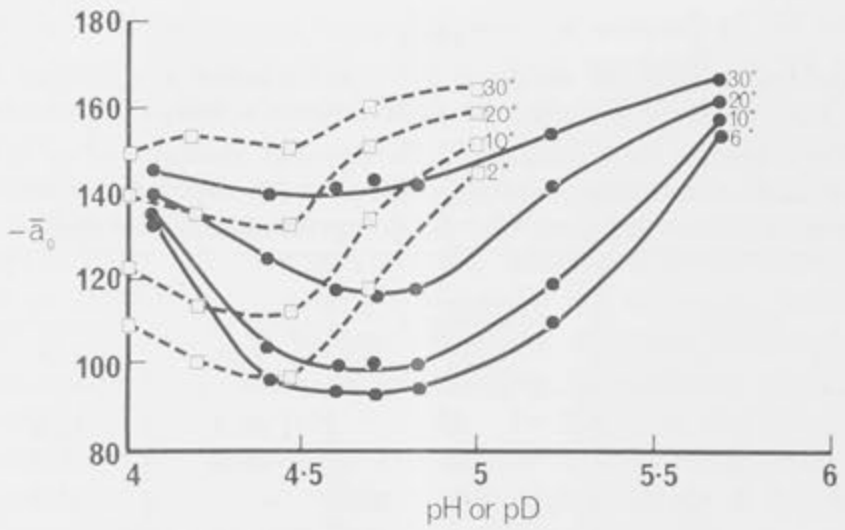


FIG. 3. The effect of temperature on the pH (or pD) dependence of the Moffitt-Yang parameter, $\bar{a}_0$. The *solid curves* refer to β-lactoglobulin A in D_2O-acetate buffers and the *broken curves* to the protein in aqueous acetate buffers.

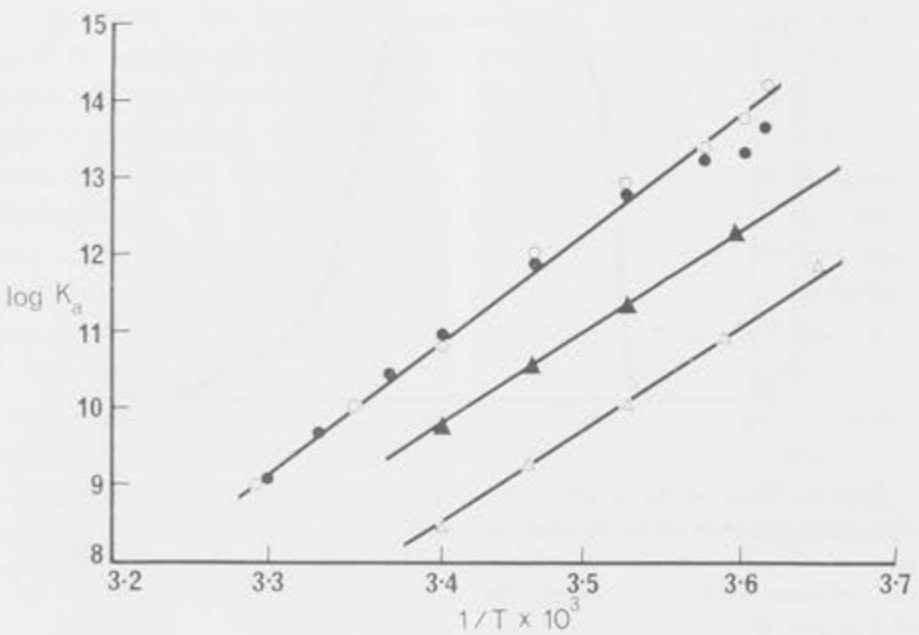


FIG. 4. A plot of log K_a values (derived from optical rotatory dispersion measurements) *versus* the reciprocal of absolute temperature. The *solid lines* were calculated by the method of least squares and the experimental *points* are as follows: ○, pD 4.65; ●, pD 4.80; △, pH 4.65; ▲, pH 4.40.

emerging D_2O gradient measured separately with a pH meter. The initial elution of protein in the aqueous phase occurred with a weight-average elution volume, $V_w(H_2O)$ = 15.5 ml referring to the corresponding plateau concentration of $\bar{c}(H_2O)$ = 0.757 g per dl. The concentration in the second plateau emerging at larger elution volumes than V' was equal to the applied concentration, $\bar{c}(D_2O)$ = 0.740 g per dl and was associated with a weight-average elution volume of 52.0 ml, which on subtraction of the loading volume, 36.9 ml, gives V_w (D_2O) = 15.1 ml. The behavior is entirely consistent with the Johnston-Ogston equation (19, 26) expressed as

$$\frac{\bar{c}(D_2O)}{\bar{c}(H_2O)} = \frac{[V' - V_w(H_2O)]}{[V' - V_w(D_2O)]} \qquad (3)$$

Thus, the concentration ratio calculated from the right-hand side of Equation 3 was 0.978 in excellent agreement with the values measured spectrophotometrically (0.977) and by the method of Lowry *et al.* (20) (0.971). The difference between V_w (H_2O) and V_w (D_2O), confirmed by the existence of two plateaux, again reflects the presence of a greater proportion of the octamer in the D_2O environment. Moreover, the experiment shows that the shift in equilibrium inherent on the change of environment occurs within the time taken to complete the elution process (approximately, 3 hours).

Optical Rotatory Dispersion—ORD spectra obtained in the range 220 to 260 nm proved to be identical regardless of whether H_2O or D_2O was used as the solvent medium. Since the experiments were conducted at 20° with solutions (0.01 g per dl) where the dimeric form is pronouncedly favored (Table I), the finding provides further evidence that the structure of the dimer is essentially the same in both environments. More information on the association reaction was derived from ORD measurements obtained at higher wave lengths with more concentrated solutions. Linear Moffitt-Yang plots (21) were obtained at all values of pH (or pD) and temperature studied and were of identical slope (−59 ± 1.5) indicating a constant helical content of 9 to 10%. This is in basic agreement with the result reported by McKenzie *et al.* (9) for studies performed at pH 4.65, although they found a slight temperature dependence of helical content (6 to 10% in the range 1–45°).

Fig. 3 presents $-\bar{a}_0$ values (intercepts of the Moffitt-Yang plots) as a function of pH (or pD). The *solid curves* (referring to the D_2O medium) exhibit a broad minimum centered at pD 4.65 to 4.80 at all temperatures studied. On the basis that $\bar{a}_0$ values may be regarded as weight-average quantities (9), it follows that the extent of association is maximal in this pD range and decreases with increasing temperature. A similar conclusion is reached on examination of the *broken curves* in Fig. 3 (referring to the aqueous medium) except that the minimum occurs at pH ∽ 4.4, a result again in basic agreement with that presented by McKenzie *et al.* (9) (their Fig. 8). Method 4 was employed to estimate log K_a values corresponding to these minima and the results are summarized in Fig. 4 in a form suitable for the determination of enthalpy changes. It is clear that the values obtained in the D_2O media in the range pD 4.65 to 4.80 are reasonably grouped about a single straight line found by least square regression. The value of the enthalpy change $\Delta H(D_2O)$, determined from the slope was −69 ± 2 kcal per mole. The results in Fig. 3 suggest that these values of log K_a should be compared with those obtained in the aqueous buffer at pH 4.4, where the apparent degree of association is maximal. It is evident from Fig. 4 that this comparison clearly shows (in agreement with Methods 1 to 3) that K_a is decreased at all temperatures in the aqueous medium. It is of interest that other methods (9, 10) have indicated that the maximum degree of association occurs near pH 4.6 in aqueous acetate buffer and, for this reason, data pertaining to this environment and calculated from the present ORD measurements have been included in Fig. 4. It is clear that the basic conclusion relating to the effect of D_2O is unaltered regardless of whether comparisons are made between results obtained at pD 4.65 and *either* pH 4.4 or 4.65. For both of the latter environments, $\Delta H(H_2O)$ = −64 ± 2 kcal per mole.

DISCUSSION

It is possible to compare the values of log K_a found by the different methods employed in this study: for example, at 12°, pH 4.65, Methods 1 to 4 yielded values of 10.0, 9.6, 10.9, and 9.8, respectively, the average value of 10.1 being in excellent agreement with that (10.0) reported by McKenzie *et al.* (9).

A similar comparison of values obtained at the same temperature in a D_2O environment (pD 4.65), *viz.* 11.3, 10.0, 12.5, and 12.4 (average 11.6), illustrates the general finding that Method 2, based on values of weight-average sedimentation coefficients gave consistently low values. This is almost certainly due to the necessity of correcting sedimentation coefficients to 20° in water by an expression (1) which assumes a linear dependence on ratios of viscosity and buoyancy terms. Nevertheless, it is clear from all methods that D_2O enhances the association of β-lactoglobulin A, a conclusion which is reached whether results are compared at a fixed pH (or pD) of 4.65 or at pH and pD values where ORD measurements indicate maximum association (Figs. 3 and 4).

A similar effect of D_2O has been observed with a variety of polymerizing protein systems, including phycocyanin (1), the protein from tobacco mosaic virus (2), glutamate dehydrogenase (3), and α-chymotrypsin (27). These systems are associated with positive enthalpy changes on polymer formation and it has been suggested (1, 27) that the indicated hydrophobic interactions are strengthened in D_2O. This interpretation is consistent with the conclusion drawn from solubility studies on nonpolar amino acids in D_2O (28) and with the observation that the critical micelle concentration of ionic detergents is smaller in D_2O than in water (28, 29). In the case of α-chymotrypsin, Aune *et al.* (27) suggest that the positive entropy change involved in dimer formation is due to the loss of "structured" water found in the vicinity of hydrophobic side chains and of charged groups in the contact region. However, the association of β-lactoglobulin A in aqueous acetate buffers (pH 4.4 to 4.65) is characterized by a large negative enthalpy change (−64 kcal per mole, Fig. 4) and with a corresponding negative entropy change (∼ −178 e.u.). Thus, hydrophobic interactions cannot be the prime forces involved in octamer formation, although they may contribute in part to it. Examination of the results found at 12° in the D_2O environment (pD 4.65, average log K_a = 11.6 and ΔH° = −69 kcal per mole) reveals that ΔF° = −15.1 kcal per mole and ΔS° = −189 e.u. These values must be regarded as approximate, due to the uncertainty in values of log K_a, but they clearly illustrate that octamer formation in the D_2O environment is associated with enthalpy and entropy changes, which are more negative than the corresponding values found in water. The same conclusion follows from calculations performed with results obtained at 4 and 20°. Therefore, it seems unlikely that the enhancement of association of β-lactoglobulin A in D_2O is due to a strengthening of any hydrophobic interactions which may contribute to octamer formation.

The alternative possibility arises that the shift in the equilibrium induced by D_2O is a direct consequence of hydrogen-deuterium exchange, since it has been postulated that deuterium bonding is of greater strength than hydrogen bonding (30). This isotope effect (31) may operate by strengthening an intermolecular hydrogen bond such as that postulated between aspartyl and glutamyl residues (11). Indirect support of the view, that intermolecular hydrogen bonding in the β-lactoglobulin A system is affected by deuterium replacement, was provided by ORD studies on the genetic variant, bovine β-lactoglobulin B. In contrast to the A variant, which possesses 2 additional aspartyl residues per mole of molecular weight 36,000, the B variant does not undergo a dimer-octamer association in aqueous acetate buffer (32) and no evidence was found that such an equilibrium was induced by the replacement of H_2O by D_2O: for example, the slight temperature dependence of $\bar{a}_0$ values found with the B variant at pD 4.65 paralleled that found in the corresponding aqueous environment (9).

Two further points in relation to the postulated importance of hydrogen-deuterium exchange merit comment. First, it is clear from Fig. 3 that the enhancement of association by D_2O is accompanied by a shift in the value of the pH (pD) corresponding to the minima, the difference being approximately 0.2 to 0.4 unit. The positions of these minima are determined partly by intermolecular interactions (probably predominantly of the hydrogen-bonding type) and partly by net electrostatic repulsive forces. It has been observed that the pK of weak acids increases on transfer from H_2O to D_2O (14, 33) and, if the ionizing groups on the protein behave similarly, this would explain (albeit qualitatively) the shift observed in Fig. 3. Secondly, the rate of hydrogen-deuterium exchange varies markedly with the group involved, but would be extremely rapid for external carboxyl groups (34). Certainly, the observation of two plateaux regions in the differential chromatography experiment (in accord with the Johnston-Ogston equation) shows that the shift in equilibrium inherent on transferring the protein from D_2O to H_2O is largely completed within the time taken to complete the elution process (∼3 hours).

In conclusion, the present results show that D_2O enhances a protein association for which the formation of polymer is accompanied by a strongly negative enthalpy change and support the view that the effect is mediated primarily as a result of hydrogen-deuterium exchange affecting both the dissociation constants of constituent groups in the protein and the strength of hydrogen (deuterium) intermolecular bonds. The findings may be related to the observed increase in thermal stability of certain proteins in D_2O (35) and to certain biological responses of deuterium inhibition, summarized by Henderson *et al.* (3). It is not suggested that deuterium oxide is without effect on hydrophobic interactions operating in *other* systems, for indeed the evidence for such an effect is compelling (1). On the other hand, in view of the present results, it seems unwise to advocate the use of deuterium oxide as a method for testing the relative importance of hydrophobic interactions (1).

REFERENCES

1. Lee, J. J., and Berns, D. S. (1968) *Biochem. J.* **110**, 465–470
2. Paglini, S., and Lauffer, M. A. (1968) *Biochemistry* **7**, 1827
3. Henderson, R. F., Henderson, T. R., and Woodfin, B. M. (1970) *J. Biol. Chem.* **245**, 3733–3737
4. Kauzmann, W. (1959) *Advan. Protein Chem.* **14**, 1
5. Reisler, E., and Eisenberg, H. (1971) *Biochemistry* **10**, 2659
6. Kumosinski, T. F., and Timasheff, S. N. (1966) *J. Amer. Chem. Soc.* **88**, 5635–5642
7. Adams, E. T., Jr., and Lewis, M. S. (1968) *Biochemistry* **7**, 1044
8. Gilbert, G. A. (1963) *Proc. Roy. Soc. London A* **276**, 354
9. McKenzie, H. A., Sawyer, W. H., and Smith, M. B. (1967) *Biochim. Biophys. Acta* **147**, 73–92
10. Townend, R., and Timasheff, S. N. (1960) *J. Amer. Chem. Soc.* **82**, 3168
11. Armstrong, J. McD., and McKenzie, H. A. (1967) *Biochim. Biophys. Acta* **147**, 93–99
12. Bell, K., and McKenzie, H. A. (1964) *Nature* **204**, 1275–1279
13. Armstrong, J. McD., McKenzie, H. A., and Sawyer, W. H. (1967) *Biochim. Biophys. Acta* **147**, 60–72
14. Glasoe, P. K., and Long, F. A. (1960) *J. Phys. Chem.* **64**, 188–191

15. Sawyer, W. H. (1966) Ph.D. Thesis, Australian National University, Canberra
16. Goldberg, R. J. (1953) *J. Phys. Chem.* **57**, 194–202
17. Kirshenbaum, I. (1951) *Physical properties and analysis of heavy water*, pp. 1–42, McGraw-Hill Book Co., New York
18. Winzor, D. J., and Scheraga, H. A. (1963) *Biochemistry* **2**, 1263
19. Baghurst, P. A., Nichol, L. W., Richards, R. J., and Winzor, D. J. (1971) *Nature* **234**, 299
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
21. Moffitt, W., and Yang, J. T. (1956) *Proc. Nat. Acad. Sci. U. S. A.* **42**, 596
22. Gilbert, G. A. (1959) *Proc. Roy. Soc. London A* **250**, 377
23. Nichol, L. W., Bethune, J. L., Kegeles, G., and Hess, E. L. (1964) in H. Neurath (Editor), *The proteins, Vol. 2*, p. 350, Academic Press, New York
24. Winzor, D. J., Loke, J. P., and Nichol, L. W. (1967) *J. Phys. Chem.* **71**, 4492
25. Andrews, P. (1970) *Methods Biochem. Anal.* **18**, 1
26. Johnston, J. P., and Ogston, A. G. (1946) *Trans. Farad. Soc.* **42**, 789
27. Aune, K. C., Goldsmith, L. C., and Timasheff, S. N. (1971) *Biochemistry* **10**, 1617
28. Kreshek, G. C., Schneider, H., and Scheraga, H. A. (1965) *J. Phys. Chem* **69**, 3132
29. Emerson, M. F., and Holtzer, A. (1967) *J. Phys. Chem.* **71**, 3320–3330
30. Némethy, G., and Scheraga, H. A. (1964) *J. Chem. Phys.* **41**, 680
31. Hattori, A., Crespi, H. L., and Katz, J. J. (1965) *Biochemistry* **4**, 1213
32. McKenzie, H. A. (1967) *Advan. Protein Chem.* **22**, 55
33. Bell, R. P. (1959) *The proton in chemistry*, p. 187, Methuen, London
34. Hvidt, A., and Nielsen, S. O. (1966) *Advan. Protein Chem.* **21**, 287
35. Hermans, J., and Scheraga, H. A. (1959) *Biochim. Biophys. Acta* **36**, 534